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METHODS FOR IDENTIFYING SMALL MOLECULES THAT BIND SPECIFIC RNA STRUCTURAL MOTIFS

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METHODS FOR IDENTIFYING SMALL MOLECULES THAT BIND SPECIFIC RNA STRUCTURAL MOTIFS

This application claims the benefit of U.S. Provisional Application No. 60/282,966, filed April 11, 2001, which is incorporated herein by reference in its entirety.

1. INTRODUCTION

The present invention relates to a method for screening and identifying test compounds that bind to a preselected target ribonucleic acid ("RNA"). Direct, non-competitive binding assays are advantageously used to screen bead-based libraries of compounds for those that selectively bind to a preselected target RNA. Binding of target RNA molecules to a particular test compound is detected using any method that measures the altered physical property of the target RNA bound to a test compound. The methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of compounds to identify pharmaceutical leads.

2. BACKGROUND OF THE INVENTION

Protein-nucleic acid interactions are involved in many cellular functions, including transcription, RNA splicing, mRNA decay, and mRNA translation. Readily accessible synthetic molecules that can bind with high affinity to specific sequences of single- or double-stranded nucleic acids have the potential to interfere with these interactions in a controllable way, making them attractive tools for molecular biology and medicine. Successful approaches for blocking function of target nucleic acids include using duplex-forming antisense oligonucleotides (Miller, 1996, Progress in Nucl. Acid Res. & Mol. Biol. 52:261-291; Ojwang & Rando, 1999, Achieving antisense inhibition by oligodeoxynucleotides containing N_7 modified 2′-deoxyguanosine using tumor necrosis factor receptor type 1, METHODS: A Companion to Methods in Enzymology 18:244-251) and peptide nucleic acids ("PNA") (Nielsen, 1999, Current Opinion in Biotechnology 10:71-75), which bind to nucleic acids via Watson-Crick base-pairing. Triplex-forming anti-gene oligonucleotides can also be designed (Ping *et al.*, 1997, RNA 3:850-860; Aggarwal *et al.*, 1996, Cancer Res. 56:5156-5164; U.S. Patent No. 5,650,316), as well as pyrrole-imidazole polyamide oligomers (Gottesfeld *et al.*, 1997, Nature 387:202-205; White *et al.*, 1998, Nature 391:468-471), which are specific for the major and minor grooves of a double helix, respectively.

In addition to synthetic nucleic acids (*i.e.*, antisense, ribozymes, and triplex-forming molecules), there are examples of natural products that interfere with deoxyribonucleic acid ("DNA") or RNA processes such as transcription or translation. For example, certain carbohydrate-based host cell factors, calicheamicin oligosaccharides, interfere with the sequence-specific binding of transcription factors to DNA and inhibit transcription *in vivo* (Ho *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:9203-9207; Liu *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:940-944). Certain classes of known antibiotics have been characterized and were found to interact with RNA. For example, the antibiotic thiostreptone binds tightly to a 60-mer from ribosomal RNA (Cundliffe *et al.*, 1990, in The Ribosome: Structure, Function & Evolution (Schlessinger *et al.*, eds.) American Society for Microbiology, Washington, D.C. pp. 479-490). Bacterial resistance to various antibiotics often involves methylation at specific rRNA sites (Cundliffe, 1989, Ann. Rev. Microbiol. 43:207-233). Aminoglycosidic aminocyclitol (aminoglycoside) antibiotics and peptide antibiotics are known to inhibit group I intron splicing by binding to specific regions of the RNA (von Ahsen *et al.*, 1991, Nature (London) 353:368-370). Some of these same aminoglycosides have also been found to inhibit hammerhead ribozyme function (Stage *et al.*, 1995, RNA 1:95-101). In addition, certain aminoglycosides and other protein synthesis inhibitors have been found to interact with specific bases in 16S rRNA (Woodcock *et al.*, 1991, EMBO J. 10:3099-3103). An oligonucleotide analog of the 16S rRNA has also been shown to interact with certain aminoglycosides (Purohit *et al.*, 1994, Nature 370:659-662). A molecular basis for hypersensitivity to aminoglycosides has been found to be located in a single base change in mitochondrial rRNA (Hutchin *et al.*, 1993, Nucleic Acids Res. 21:4174-4179). Aminoglycosides have also been shown to inhibit the interaction between specific structural RNA motifs and the corresponding RNA binding protein. Zapp *et al.* (Cell, 1993, 74:969-978) has demonstrated that the aminoglycosides neomycin B, lividomycin A, and tobramycin can block the binding of Rev, a viral regulatory protein required for viral gene expression, to its viral recognition element in the IIB (or RRE) region of HIV RNA. This blockage appears to be the result of competitive binding of the antibiotics directly to the RRE RNA structural motif.

Single stranded sections of RNA can fold into complex tertiary structures consisting of local motifs such as loops, bulges, pseudoknots, guanosine quartets and turns (Chastain & Tinoco, 1991, Progress in Nucleic Acid Res. & Mol. Biol. 41:131-177; Chow & Bogdan, 1997, Chemical Reviews 97:1489-1514; Rando & Hogan, 1998, Biologic activity of guanosine quartet forming oligonucleotides in "Applied Antisense Oligonucleotide Technology" Stein. & Krieg (eds) John Wiley and Sons, New York, pages 335-352). Such

structures can be critical to the activity of the nucleic acid and affect functions such as regulation of mRNA transcription, stability, or translation (Weeks & Crothers, 1993, Science 261:1574-1577). The dependence of these functions on the native three-dimensional structural motifs of single-stranded stretches of nucleic acids makes it difficult to identify or design synthetic agents that bind to these motifs using general, simple-to-use sequence-specific recognition rules for the formation of double- and triple-helical nucleic acids used in the design of antisense and ribozyme type molecules. Approaches to screening generally involve competitive assays designed to identify compounds that disrupt the interaction between a target RNA and a physiological, host cell factor(s) that had been previously identified to specifically interact with that particular target RNA. In general, such assays require the identification and characterization of the host cell factor(s) deemed to be required for the function of the target RNA. Both the target RNA and its preselected host cell binding partner are used in a competitive format to identify compounds that disrupt or interfere with the two components in the assay.

Citation or identification of any reference in Section 2 of this application is not an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to methods for identifying compounds that bind to preselected target elements of nucleic acids including, but not limited to, specific RNA sequences, RNA structural motifs, and/or RNA structural elements. The specific target RNA sequences, RNA structural motifs, and/or RNA structural elements are used as targets for screening small molecules and identifying those that directly bind these specific sequences, motifs, and/or structural elements. For example, methods are described in which a preselected target RNA having a detectable label is used to screen a library of test compounds, preferably under physiologic conditions. Any complexes formed between the target RNA and a member of the library are identified using methods that detect the labeled target RNA bound to a test compound. In particular, the present invention relates to methods for using a target RNA having a detectable label to screen a bead-based library of test compounds. Compounds in the bead-based library that bind to the labeled target RNA will form a bead-based detectably labeled complex, which can be separated from the unbound beads and unbound target RNA in the liquid phase by a number of physical means, including, but not limited to, flow cytometry, affinity chromatography, manual batch mode separation, suspension of beads in electric fields, and microwave of the bead-based detectably labeled complex. The detectably labeled complex can then be identified by the label on the target

RNA and removed from the uncomplexed, unlabeled test compounds in the library. The structure of the test compound complexed with the labeled RNA is then ascertained by *de novo* structure determination of the test compounds using, for example, mass spectrometry or nuclear magnetic resonance ("NMR"). The test compounds identified are useful for any purpose to which a binding reaction may be put, for example in assay methods, diagnostic procedures, cell sorting, as inhibitors of target molecule function, as probes, as sequestering agents and the like. In addition, small organic molecules which interact specifically with target RNA molecules may be useful as lead compounds for the development of therapeutic agents.

The methods described herein for the identification of compounds that directly bind to a particular preselected target RNA are well suited for high-throughput screening. The direct binding method of the invention offers advantages over drug screening systems for competitors that inhibit the formation of naturally-occurring RNA binding protein:target RNA complexes; *i.e.*, competitive assays. The direct binding method of the invention is rapid and can be set up to be readily performed, *e.g.*, by a technician, making it amenable to high throughput screening. The method of the invention also eliminates the bias inherent in the competitive drug screening systems, which require the use of a preselected host cell factor that may not have physiological relevance to the activity of the target RNA. Instead, the methods of the invention are used to identify any compound that can directly bind to specific target RNA sequences, RNA structural motifs, and/or RNA structural elements, preferably under physiologic conditions. As a result, the compounds so identified can inhibit the interaction of the target RNA with any one or more of the native host cell factors (whether known or unknown) required for activity of the RNA *in vivo*.

The present invention may be understood more fully by reference to the detailed description and examples, which are intended to illustrate non-limiting embodiments of the invention.

3.1. Definitions

As used herein, a "target nucleic acid" refers to RNA, DNA, or a chemically modified variant thereof. In a preferred embodiment, the target nucleic acid is RNA. A target nucleic acid also refers to tertiary structures of the nucleic acids, such as, but not limited to loops, bulges, pseudoknots, guanosine quartets and turns. A target nucleic acid also refers to RNA elements such as, but not limited to, the HIV TAR element, internal ribosome entry site, "slippery site", instability elements, and adenylate uridylate-rich

elements, which are described in Section 4.1. Non-limiting examples of target nucleic acids are presented in Section 4.1 and Section 5.

As used herein, a "library" refers to a plurality of test compounds with which a target nucleic acid molecule is contacted. A library can be a combinatorial library, *e.g.*, a collection of test compounds synthesized using combinatorial chemistry techniques, or a collection of unique chemicals of low molecular weight (less than 1000 daltons) that each occupy a unique three-dimensional space.

As used herein, a "label" or "detectable label" is a composition that is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes (*e.g.*, ^{32}P, ^{35}S, and ^{3}H), dyes, fluorescent dyes, electron-dense reagents, enzymes and their substrates (*e.g.*, as commonly used in enzyme-linked immunoassays, *e.g.*, alkaline phosphatase and horse radish peroxidase), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available. Moreover, a label or detectable moiety can include an "affinity tag" that, when coupled with the target nucleic acid and incubated with a test compound or compound library, allows for the affinity capture of the target nucleic acid along with molecules bound to the target nucleic acid. One skilled in the art will appreciate that a affinity tag bound to the target nucleic acids has, by definition, a complimentary ligand coupled to a solid support that allows for its capture. For example, useful affinity tags and complimentary ligands include, but are not limited to, biotin-streptavidin, complimentary nucleic acid fragments (e.g., oligo dT-oligo dA, oligo T-oligo A, oligo dG-oligo dC, oligo G-oligo C), aptamer complexes, or haptens and proteins for which antisera or monoclonal antibodies are available. The label or detectable moiety is typically bound, either covalently, through a linker or chemical bound, or through ionic, van der Waals or hydrogen bonds to the molecule to be detected.

As used herein, a "dye" refers to a molecule that, when exposed to radiation, emits radiation at a level that is detectable visually or via conventional spectroscopic means. As used herein, a "visible dye" refers to a molecule having a chromophore that absorbs radiation in the visible region of the spectrum (*i.e.*, having a wavelength of between about 400 nm and about 700 nm) such that the transmitted radiation is in the visible region and can be detected either visually or by conventional spectroscopic means. As used herein, an "ultraviolet dye" refers to a molecule having a chromophore that absorbs radiation in the ultraviolet region of the spectrum (*i.e.*, having a wavelength of between about 30 nm and about 400 nm). As used herein, an "infrared dye" refers to a molecule having a chromophore that absorbs radiation in the infrared region of the spectrum (*i.e.*, having a wavelength

between about 700 nm and about 3,000 nm). A "chromophore" is the network of atoms of the dye that, when exposed to radiation, emits radiation at a level that is detectable visually or via conventional spectroscopic means. One of skill in the art will readily appreciate that although a dye absorbs radiation in one region of the spectrum, it may emit radiation in another region of the spectrum. For example, an ultraviolet dye may emit radiation in the visible region of the spectrum. One of skill in the art will also readily appreciate that a dye can transmit radiation or can emit radiation via fluorescence or phosphorescence.

The phrase "pharmaceutically acceptable salt(s)," as used herein includes but is not limited to salts of acidic or basic groups that may be present in test compounds identified using the methods of the present invention. Test compounds that are basic in nature are capable of forming a wide variety of salts with various inorganic and organic acids. The acids that can be used to prepare pharmaceutically acceptable acid addition salts of such basic compounds are those that form non-toxic acid addition salts, *i.e.*, salts containing pharmacologically acceptable anions, including but not limited to sulfuric, citric, maleic, acetic, oxalic, hydrochloride, hydrobromide, hydroiodide, nitrate, sulfate, bisulfate, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Test compounds that include an amino moiety may form pharmaceutically or cosmetically acceptable salts with various amino acids, in addition to the acids mentioned above. Test compounds that are acidic in nature are capable of forming base salts with various pharmacologically or cosmetically acceptable cations. Examples of such salts include alkali metal or alkaline earth metal salts and, particularly, calcium, magnesium, sodium lithium, zinc, potassium, and iron salts.

By "substantially one type of test compound," as used herein, is meant that the assay can be performed in such a fashion that at some point, only one compound need be used in each reaction so that, if the result is indicative of a binding event occurring between the target RNA molecule and the test compound the test compound, can be easily identified.

4. <u>DETAILED DESCRIPTION OF THE INVENTION</u>

The present invention relates to methods for identifying compounds that bind to preselected target elements of nucleic acids, in particular, RNAs, including but not limited to preselected target RNA sequencing structural motifs, or structural elements. Methods are described in which a preselected target RNA having a detectable label is used to screen a

library of test compounds. Any complexes formed between the target RNA and a member of the library are identified using methods that detect the labeled target RNA bound to a test compound. In particular, the present invention relates to methods for using a target RNA having a detectable label to screen a bead-based library of test compounds. Compounds in the bead-based library that bind to the labeled target RNA will form a bead-based detectably labeled complex, which can be separated from the unbound target RNA in the liquid phase by a number of physical means, such as, but not limited to, flow cytometry, affinity chromatography, manual batch mode separation, suspension of beads in electric fields, and microwave of the bead-based detectably labeled complex. The detectably labeled complex can then be identified by the label on the target RNA and removed from the uncomplexed, unlabeled test compounds in the library. The structure of the test compound attached to the labeled RNA is then ascertained by *de novo* structure determination of the test compounds using, for example, mass spectrometry or nuclear magnetic resonance ("NMR").

Thus, the methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of test compounds, in which the test compounds of the library that specifically bind a preselected target nucleic acid are easily distinguished from non-binding members of the library. The structures of the binding molecules are ascertained by *de novo* structure determination of the test compounds using, for example, mass spectrometry or nuclear magnetic resonance ("NMR"). The test compounds so identified are useful for any purpose to which a binding reaction may be put, for example in assay methods, diagnostic procedures, cell sorting, as inhibitors of target molecule function, as probes, as sequestering agents and lead compounds for development of therapeutics, and the like. Small organic compounds that are identified to interact specifically with the target RNA molecules are particularly attractive candidates as lead compounds for the development of therapeutic agents.

The assay of the invention reduces bias introduced by competitive binding assays which require the identification and use of a host cell factor (presumably essential for modulating RNA function) as a binding partner for the target RNA. The assays of the present invention are designed to detect any compound or agent that binds to the target RNA, preferably under physiologic conditions. Such agents can then be tested for biological activity, without establishing or guessing which host cell factor or factors is required for modulating the function and/or activity of the target RNA.

Section 4.1 describes examples of protein-RNA interactions that are important in a variety of cellular functions and several target RNA elements that can be used to identify test compounds. Compounds that inhibit these interactions by binding to the RNA and

successfully competing with the natural protein or host cell factor that endogenously binds to the RNA may be important, *e.g.*, in treating or preventing a disease or abnormal condition, such as an infection or unchecked growth. Section 4.2 describes detectable labels for target nucleic acids that are useful in the methods of the invention. Section 4.3 describes libraries of test compounds. Section 4.4 provides conditions for binding a labeled target RNA to a test compound of a library and detecting RNA binding to a test compound using the methods of the invention. Section 4.5 provides methods for separating complexes of target RNAs bound to a test compound from an unbound RNA. Section 4.6 describes methods for identifying test compounds that are bound to the target RNA. Section 4.7 describes a secondary, biological screen of test compounds identified by the methods of the invention to test the effect of the test compounds *in vivo*. Section 4.8 describes the use of test compounds identified by the methods of the invention for treating or preventing a disease or abnormal condition in mammals.

4.1. Biologically Important RNA-Host Cell Factor Interactions

Nucleic acids, and in particular RNAs, are capable of folding into complex tertiary structures that include bulges, loops, triple helices and pseudoknots, which can provide binding sites for host cell factors, such as proteins and other RNAs. RNA-protein and RNA-RNA interactions are important in a variety cellular functions, including transcription, RNA splicing, RNA stability and translation. Furthermore, the binding of such host cell factors to RNAs may alter the stability and translational efficiency of such RNAs, and according affect subsequent translation. For example, some diseases are associated with protein overproduction or decreased protein function. In this case, the identification of compounds to modulate RNA stability and translational efficiency will be useful to treat and prevent such diseases.

The methods of the present invention are useful for identifying test compounds that bind to target RNA elements in a high throughput screening assay of libraries of test compounds in solution. In particular, the methods of the present invention are useful for identifying a test compound that binds to a target RNA elements and inhibits the interaction of that RNA with one or more host cell factors *in vivo*. The molecules identified using the methods of the invention are useful for inhibiting the formation of a specific bound RNA:host cell factor complexes *in vivo*.

In some embodiments, test compounds identified by the methods of the invention are useful for increasing or decreasing the translation of messenger RNAs ("mRNAs"), *e.g.*, protein production, by binding to one or more regulatory elements in the 5'

untranslated region, the 3' untranslated region, or the coding region of the mRNA. Compounds that bind to mRNA can, *inter alia*, increase or decrease the rate of mRNA processing, alter its transport through the cell, prevent or enhance binding of the mRNA to ribosomes, suppressor proteins or enhancer proteins, or alter mRNA stability. Accordingly, compounds that increase or decrease mRNA translation can be used to treat or prevent disease. For example, diseases associated with protein overproduction, such as amyloidosis, or with the production of mutant proteins, such as *Ras*, can be treated or prevented by decreasing translation of the mRNA that codes for the overproduced protein, thus inhibiting production of the protein. Conversely, the symptoms of diseases associated with decreased protein function, such as hemophelia, may be treated by increasing translation of mRNA coding for the protein whose function is decreased, *e.g.*, factor IX in some forms of hemophilia.

The methods of the invention can be used to identify compounds that bind to mRNAs coding for a variety of proteins with which the progression of diseases in mammals is associated. These mRNAs include, but are not limited to, those coding for amyloid protein and amyloid precursor protein; anti-angiogenic proteins such as angiostatin, endostatin, METH-1 and METH-2; apoptosis inhibitor proteins such as survivin, clotting factors such as Factor IX, Factor VIII, and others in the clotting cascade; collagens; cyclins and cyclin inhibitors, such as cyclin dependent kinases, cyclin D1, cyclin E, WAF1, cdk4 inhibitor, and MTS1; cystic fibrosis transmembrane conductance regulator gene (CFTR); cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17 and other interleukins; hematopoetic growth factors such as erythropoietin (Epo); colony stimulating factors such as G-CSF, GM-CSF, M-CSF, SCF and thrombopoietin; growth factors such as BNDF, BMP, GGRP, EGF, FGF, GDNF, GGF, HGF, IGF-1, IGF-2, KGF, myotrophin, NGF, OSM, PDGF, somatotrophin, TGF-ß, TGF-α and VEGF; antiviral cytokines such as interferons, antiviral proteins induced by interferons, TNF-α, and TNF-ß; enzymes such as cathepsin K, cytochrome P-450 and other cytochromes, farnesyl transferase, glutathione-s transferases, heparanase, HMG CoA synthetase, N-acetyltransferase, phenylalanine hydroxylase, phosphodiesterase, ras carboxyl-terminal protease, telomerase and TNF converting enzyme; glycoproteins such as cadherins, *e.g.*, N-cadherin and E-cadherin; cell adhesion molecules; selectins; transmembrane glycoproteins such as CD40; heat shock proteins; hormones such as 5-α reductase, atrial natriuretic factor, calcitonin, corticotrophin releasing factor, diuretic hormones, glucagon, gonadotropin, gonadotropin releasing hormone, growth hormone, growth hormone releasing factor, somatotropin, insulin, leptin, luteinizing hormone, luteinizing hormone releasing hormone,

parathyroid hormone, thyroid hormone, and thyroid stimulating hormone; proteins involved in immune responses, including antibodies, CTLA4, hemagglutinin, MHC proteins, VLA-4, and kallikrein-kininogen-kinin system; ligands such as CD4; oncogene products such as *sis, hst,* protein tyrosine kinase receptors, *ras, abl, mos, myc, fos, jun, H-ras, ki-ras, c-fms, bcl-2, L-myc, c-myc, gip, gsp,* and *HER-2*; receptors such as bombesin receptor, estrogen receptor, GABA receptors, growth factor receptors including EGFR, PDGFR, FGFR, and NGFR, GTP-binding regulatory proteins, interleukin receptors, ion channel receptors, leukotriene receptor antagonists, lipoprotein receptors, opioid pain receptors, substance P receptors, retinoic acid and retinoid receptors, steroid receptors, T-cell receptors, thyroid hormone receptors, TNF receptors; tissue plasminogen activator; transmembrane receptors; transmembrane transporting systems, such as calcium pump, proton pump, Na/Ca exchanger, MRP1, MRP2, P170, LRP, and cMOAT; transferrin; and tumor suppressor gene products such as *APC, brca1, brca2, DCC, MCC, MTS1, NF1, NF2, nm23, p53* and *Rb.* In addition to the eukaryotic genes listed above, the invention, as described, can be used to define molecules that interrupt viral, bacterial or fungal transcription or translation efficiencies and therefore form the basis for a novel anti-infectious disease therapeutic. Other target genes include, but are not limited to, those disclosed in Section 4.1 and Section 5.

The methods of the invention can be used to identify mRNA-binding test compounds for increasing or decreasing the production of a protein, thus treating or preventing a disease associated with decreasing or increasing the production of said protein, respectively. The methods of the invention may be useful for identifying test compounds for treating or preventing a disease in mammals, including cats, dogs, swine, horses, goats, sheep, cattle, primates and humans. Such diseases include, but are not limited to, amyloidosis, hemophilia, Alzheimer's disease, atherosclerosis, cancer, giantism, dwarfism, hypothyroidism, hyperthyroidism, inflammation, cystic fibrosis, autoimmune disorders, diabetes, aging, obesity, neurodegenerative disorders, and Parkinson's disease. Other diseases include, but are not limited to, those described in Section 4.1 and diseases caused by aberrant expression of the genes disclosed in Example 5. In addition to the eukaryotic genes listed above, the invention, as described, can be used to define molecules that interrupt viral, bacterial or fungal transcription or translation efficiencies and therefore form the basis for a novel anti-infectious disease therapeutic.

In other embodiments, test compounds identified by the methods of the invention are useful for preventing the interaction of an RNA, such as a transfer RNA ("tRNA"), an enzymatic RNA or a ribosomal RNA ("rRNA"), with a protein or with another RNA, thus preventing, *e.g.*, assembly of an *in vivo* protein-RNA or RNA-RNA complex that

is essential for the viability of a cell. The term "enzymatic RNA," as used herein, refers to RNA molecules that are either self-splicing, or that form an enzyme by virtue of their association with one or more proteins, *e.g.,* as in RNase P, telomerase or small nuclear ribonuclear protein particles. For example, inhibition of an interaction between rRNA and one or more ribosomal proteins may inhibit the assembly of ribosomes, rendering a cell incapable of synthesizing proteins. In addition, inhibition of the interaction of precursor rRNA with ribonucleases or ribonucleoprotein complexes (such as RNase P) that process the precursor rRNA prevent maturation of the rRNA and its assembly into ribosomes. Similarly, a tRNA:tRNA synthetase complex may be inhibited by test compounds identified by the methods of the invention such that tRNA molecules do not become charged with amino acids. Such interactions include, but are not limited to, rRNA interactions with ribosomal proteins, tRNA interactions with tRNA synthetase, RNase P protein interactions with RNase P RNA, and telomerase protein interactions with telomerase RNA.

In other embodiments, test compounds identified by the methods of the invention are useful for treating or preventing a viral, bacterial, protozoan or fungal infection. For example, transcriptional up-regulation of the genes of human immunodeficiency virus type 1 ("HIV-1") requires binding of the HIV Tat protein to the HIV trans-activation response region RNA ("TAR RNA"). HIV TAR RNA is a 59-base stem-loop structure located at the 5'-end of all nascent HIV-1 transcripts (Jones & Peterlin, 1994, Annu. Rev. Biochem. 63:717-43). Tat protein is known to interact with uracil 23 in the bulge region of the stem of TAR RNA. Thus, TAR RNA is a potential binding target for test compounds, such as small peptides and peptide analogs that bind to the bulge region of TAR RNA and inhibit formation of a Tat-TAR RNA complex involved in HIV-1 upregulation (see Hwang *et al.*,1999 Proc. Natl. Acad. Sci. USA 96:12997-13002). Accordingly, test compounds that bind to TAR RNA are useful as anti-HIV therapeutics (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Hamy *et al.*, 1998, Biochemistry 37:5086-5095; Mei *et al.*, 1998, Biochemistry 37:14204-14212), and therefore, are useful for treating or preventing AIDS.

The methods of the invention can be used to identify test compounds to treat or prevent viral, bacterial, protozoan or fungal infections in a patient. In some embodiments, the methods of the invention are useful for identifying compounds that decrease translation of microbial genes by interacting with mRNA, as described above, or for identifying compounds that inhibit the interactions of microbial RNAs with proteins or other ligands that are essential for viability of the virus or microbe. Examples of microbial target RNAs useful in the present invention for identifying antiviral, antibacterial, anti-protozoan and anti-fungal compounds include, but are not limited to, general antiviral and anti-inflammatory targets

such as mRNAs of INFα, INFγ, RNAse L, RNAse L inhibitor protein, PKR, tumor necrosis factor, interleukins 1-15, and IMP dehydrogenase; internal ribosome entry sites; HIV-1 CT rich domain and RNase H mRNA; HCV internal ribosome entry site (required to direct translation of HCV mRNA), and the 3'-untranslated tail of HCV genomes; rotavirus NSP3 binding site, which binds the protein NSP3 that is required for rotavirus mRNA translation; HBV epsilon domain; Dengue virus 5' and 3' untranslated regions, including IRES; INFα, INFβ and INFγ; plasmodium falciparum mRNAs; the 16S ribosomal subunit ribosomal RNA and the RNA component of RNase P of bacteria; and the RNA component of telomerase in fungi and cancer cells. Other target viral and bacterial mRNAs include, but are not limited to, those disclosed in Section 5.

One of skill in the art will appreciate that, although such target RNAs are functionally conserved in various species (*e.g.*, from yeast to humans), they exhibit nucleotide sequence and structural diversity. Therefore, inhibition of, for example, yeast telomerase by an anti-fungal compound identified by the methods of the invention might not interfere with human telomerase and normal human cell proliferation.

Thus, the methods of the invention can be used to identify test compounds that interfere with one or more target RNA interactions with host cell factors that are important for cell growth or viability, or essential in the life cycle of a virus, a bacterium, a protozoa or a fungus. Such test compounds and/or congeners that demonstrate desirable biologic and pharmacologic activity can be administered to a patient in need thereof in order to treat or prevent a disease caused by viral, bacterial, protozoan, or fungal infections. Such diseases include, but are not limited to, HIV infection, AIDS, human T-cell leukemia, SIV infection, FIV infection, feline leukemia, hepatitis A, hepatitis B, hepatitis C, Dengue fever, malaria, rotavirus infection, severe acute gastroenteritis, diarrhea, encephalitis, hemorrhagic fever, syphilis, legionella, whooping cough, gonorrhea, sepsis, influenza, pneumonia, tinea infection, candida infection, and meningitis.

Non-limiting examples of RNA elements involved in the regulation of gene expression, *i.e.*, mRNA stability, translational efficiency via translational initiation and ribosome assembly, *etc.*, include the HIV TAR element, internal ribosome entry site, "slippery site", instability elements, and adenylate uridylate-rich elements, as discussed below.

4.1.1. HIV TAR Element

Transcriptional up-regulation of the genes of human immunodeficiency virus type 1 ("HIV-1") requires binding of the HIV Tat protein to the HIV trans-activation

response region RNA ("TAR RNA"), a 59-base stem-loop structure located at the 5' end of all nascent HIV-1 transcripts (Jones & Peterlin, 1994, Annu. Rev. Biochem. 63:717-43). Tat protein is known to interact with uracil 23 in the bulge region of the stem of TAR RNA. Thus, TAR RNA is a useful binding target for test compounds, such as small peptides and peptide analogs that bind to the bulge region of TAR RNA and inhibit formation of a Tat-TAR RNA complex involved in HIV-1 up-regulation (see Hwang *et al.*,1999 Proc. Natl. Acad. Sci. USA 96:12997-13002). Accordingly, test compounds that bind to TAR RNA can be useful as anti-HIV therapeutics (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Hamy *et al.*, 1998, Biochemistry 37:5086-5095; Mei *et al.*, 1998, Biochemistry 37:14204-14212), and therefore, are useful for treating or preventing AIDS.

4.1.2. <u>Internal Ribosome Entry Site ("IRES")</u>

Internal ribosome entry sites ("IRES") are found in the 5' untranslated regions ("5' UTR") of several mRNAs, and are thought to be involved in the regulation of translational efficiency. When the IRES element is present on an mRNA downstream of a translational stop codon, it directs ribosomal re-entry (Ghattas *et al.*, 1991, Mol. Cell. Biol. 11:5848-5959), which permits initiation of translation at the start of a second open reading frame.

As reviewed by Jang *et al.*, a large segment of the 5' nontranslated region, approximately 400 nucleotides in length, promotes internal entry of ribosomes independent of the non-capped 5' end of picornavirus mRNAs (mammalian plus-strand RNA viruses whose genomes serve as mRNA). This 400 nucleotide segment (IRES), maps approximately 200 nt down-stream from the 5' end and is highly structured. IRES elements of different picornaviruses, although functionally similar *in vitro* and *in vivo*, are not identical in sequence or structure. However, IRES elements of the genera entero- and rhinoviruses, on the one hand, and cardio- and aphthoviruses, on the other hand, reveal similarities corresponding to phylogenetic kinship. All IRES elements contain a conserved Yn-Xm-AUG unit (Y, pyrimidine; X, nucleotide) which appears essential for IRES function. The IRES elements of cardio-, entero- and aphthoviruses bind a cellular protein, p57. In the case of cardioviruses, the interaction between a specific stem-loop of the IREs is essential for translation in vitro. The IRES elements of entero- and cardioviruses also bind the cellular protein, p52, but the significance of this interaction remains to be shown. The function of p57 or p52 in cellular metabolism is unknown. Since picornaviral IRES elements function *in vivo* in the absence of any viral gene products, is speculated that IRES-like elements may also occur in specific cellular mRNAs releasing them from cap-dependent translation (Jang *et al.*,

1990, Enzyme 44(1-4):292-309).

4.1.3. "Slippery Site"

Programmed, or directed, ribosomal frameshifting, when ribosomes shift from one translation reading frame to another and synthesize two viral proteins from a single viral mRNA, is directed by a unique site in viral mRNAs called the "slippery site." The slippery site directs ribosomal frameshifting in the -1 or +1 direction that causes the ribosome to slip by one base in the 5' direction thereby placing the ribosome in the new reading frame to produce a new protein.

Programmed, or directed, ribosomal frameshifting is of particular value to viruses that package their plus strands, as it eliminates the need to splice their mRNAs and reduces the risk of packaging defective genomes and regulates the ratio of viral proteins synthesized. Examples of programmed translational frameshifting (both +1 and -1 shifts) have been identified in ScV systems (Lopinski *et al.*, 2000, Mol. Cell. Biol. 20(4):1095-103, retroviruses (Falk *et al.*, 1993, J. Virol. 67:273-6277; Jacks & Varmus, 1985, Science 230:1237-1242; Morikawa & Bishop, 1992, Virology 186:389-397; Nam *et al.*, 1993, J. Virol. 67:196-203); coronaviruses (Brierley *et al.*, 1987, EMBO J. 6:3779-3785; Herold & Siddell, 1993, Nucleic Acids Res. 21:5838-5842); giardiaviruses, which are also members of the Totiviridae (Wang *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:8595-8599); two bacterial genes (Blinkowa & Walker, 1990, Nucleic Acids Res., 18:1725-1729; Craigen & Caskey, 1986, Nature 322:273); bacteriophage genes (Condron *et al.*, 1991, Nucleic Acids Res. 19:5607-5612); astroviruses (Marczinke *et al.*, 1994, J. Virol. 68:5588-5595); the yeast EST3 gene (Lundblad & Morris, 1997, Curr. Biol. 7:969-976); and the rat, mouse, Xenopus, and Drosophila ornithine decarboxylase antizymes (Matsufuji *et al.*, 1995, Cell 80:51-60); and a significant number of cellular genes (Herold & Siddell, 1993, Nucleic Acids Res. 21:5838-5842).

Drugs targeted to ribosomal frameshifting minimize the problem of virus drug resistance because this strategy targets a host cellular process rather than one introduced into the cell by the virus, which minimizes the ability of viruses to evolve drug-resistant mutants. Compounds that target the RNA elements involved in regulating programmed frameshifting should have several advantages, including (a) any selective pressure on the host cellular translational machinery to adapt to the drugs would have to occur at the host evolutionary time scale, which is on the order of millions of years, (b) ribosomal frameshifting is not used to express any host proteins, and (c) altering viral frameshifting efficiencies by modulating

the activity of a host protein minimizing the likelihood that the virus will acquire resistance to such inhibition by mutations in its own genome.

4.1.4. **Instability Elements**

"Instability elements" may be defined as specific sequence elements that promote the recognition of unstable mRNAs by cellular turnover machinery. Instability elements have been found within mRNA protein coding regions as well as untranslated regions.

Altering the control of stability of normal mRNAs may lead to disease. The alteration of mRNA stability has been implicated in diseases such as, but not limited to, cancer, immune disorders, heart disease, and fibrotic disorders.

There are several examples of mutations that delete instability elements which then result in stabilization of mRNAs that may be involved in the onset of cancer. In Burkitt's lymphoma, a portion of the c-*myc* proto-oncogene is translocated to an Ig locus, producing a form of the c-*myc* mRNA that is five times more stable (*see*, *e.g.*, Kapstein *et al.*, 1996, J. Biol. Chem. 271(31):18875-84). The highly oncogenic v-*fos* mRNA lacks the 3' UTR adenylate uridylate rich element ("ARE") that is found in the more labile and weakly oncogenic c-*fos* mRNA (*see*, *e.g.*, Schiavi *et al.*, 1992, Biochim Biophys Acta. 1114(2-3):95-106). Differences between the benign cervical lesions brought about by nonintegrated circular human papillomavirus type 16 and its integrated form, that lacks the 3' UTR ARE and correlates with cervical carcinomas, may be a consequence of stabilizing the E6/E7 transcripts encoding oncogenic proteins. Integration of the virus results in deletion of the ARE instability element, resulting in stabilizion of the transcripts and over-expression of the proteins (*see*, *e.g.*, Jeon & Lambert, 1995, Proc. Natl. Acad. Sci. USA 92(5):1654-8). Deletion of AREs from the 3' UTR of the IL-2 and IL-3 genes promotes increased stabilization of these mRNAs, high expression of these proteins, and leads to the formation of cancerous cells (*see*, *e.g.*, Stoecklin *et al.*, 2000, Mol. Cell. Biol. 20(11):3753-63).

Mutations in trans-acting factors involved in mRNA turnover may also promote cancer. In monocytic tumors, the lymphokine GM-CSF mRNA is specifically stabilized as a consequence of an oncogenic lesion in a trans-acting factor that controls mRNA turnover rates. Furthermore, the normally unstable IL-3 transcript is inappropriately long-lived in mast tumor cells. Similarly, the labile GM-CSF mRNA is greatly stabilized in bladder carcinoma cells. *See, e.g.*, Bickel *et al.*, 1990, J. Immunol. 145(3):840-5.

The immune system is regulated by a large number of regulatory molecules that either activate or inhibit the immune response. It has now been clearly demonstrated that

stability of the transcripts encoding these proteins are highly regulated. Altered regulation of these molecules leads to mis-regulation of this process and can result in drastic medical consequences. For example, recent results using transgenic mice have shown that mis-regulation of the stability of the important modulator TNFα mRNA leads to diseases such as, but not limited to, rheumatoid arthritis and a Crohn's-like liver disease. *See, e.g.*, Clark, 2000, Arthritis Res. 2(3):172-4.

Smooth muscle in the heart is modulated by the β-adrenergic receptor, which in turn responds to the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Chronic heart failure is characterized by impairment of smooth muscle cells, which results, in part, from the more rapid decay of the β-adrenergic receptor mRNA. *See, e.g.*, Ellis & Frielle T., 1999, Biochem. Biophys. Res. Commun. 258(3):552-8.

A large number of diseases result from over-expression of collagen. For example, cirrhosis results from damage to the liver as a consequence of cancer, viral infection, or alcohol abuse. Such damage causes mis-regulation of collagen expression, leading to the formation of large collagen deposits. Recent results indicate that the sizeable increase in collagen expression is largely attributable to stabilization of its mRNA. *See, e.g.*, Lindquist *et al.*, 2000, Am. J. Physiol. Gastrointest. Liver Physiol. 279(3):G471-6.

4.1.5. Adenylate Uridylate-rich Elements ("ARE")

Adenylate uridylate-rich elements ("ARE") are found in the 3' untranslated regions ("3' UTR") of several mRNAs, and involved in the turnover of mRNAs, such as but not limited to transcription factors, cytokines, and lymphokines. AREs may function both as stabilizing and destabilizing elements. ARE mRNAs are classified into five groups, depending on sequence (Bakheet *et al.*, 2001, Nucl. Acids Res. 29(1):246-254). An ongoing database at the web site http://rc.kfshrc.edu.sa/ared contains ARE-containing mRNAs and their cluster groups, which is incorporated by reference in its entirety. The ARE motifs are classified as follows:

Group I Cluster	(AUUUAUUUAUUUAUUUAUUUA)	SEQ ID NO: 1
Group II Cluster	(AUUUAUUUAUUUAUUUA) stretch	SEQ ID NO: 2
Group III Cluster	(WAUUUAUUUAUUUAW) stretch	SEQ ID NO: 3
Group IV Cluster	(WWAUUUAUUUAWW) stretch	SEQ ID NO: 4
Group V Cluster	(WWWWAUUUAWWWW) stretch	SEQ ID NO: 5

The ARE-mRNAs were clustered into five groups containing five, four, three and two pentameric repeats, while the last group contains only one pentamer within the

13-bp ARE pattern. Functional categories were assigned whenever possible according to NCBI-COG functional annotation (Tatusov *et al.*, 2001, Nucleic Acids Research, 29(1): 22-28), in addition to the categories: inflammation, immune response, development/differentiation, using an extensive literature search.

Group I contains many secreted proteins including GM-CSF, IL-1, IL-11, IL-12 and Gro-ß that affect the growth of hematopoietic and immune cells (Witsell & Schook, 1992, Proc. Natl Acad. Sci. USA, 89:4754–4758). Although TNFα is both a pro-inflammatory and anti-tumor protein, there is experimental evidence that it can act as a growth factor in certain leukemias and lymphomas (Liu *et al.*, 2000, J. Biol. Chem. 275:21086–21093).

Unlike Group I, Groups II–V contain functionally diverse gene families comprising immune response, cell cycle and proliferation, inflammation and coagulation, angiogenesis, metabolism, energy, DNA binding and transcription, nutrient transportation and ionic homeostasis, protein synthesis, cellular biogenesis, signal transduction, and apoptosis (Bakheet *et al.*, 2001, Nucl. Acids Res. 29(1):246-254).

Several groups have described ARE-binding proteins that influence the ARE-mRNA stability. Among the well-characterized proteins are the mammalian homologs of ELAV (embryonic lethal abnormal vision) proteins including AUF1, HuR and Hel-N2 (Zhang *et al.*, 1993, Mol. Cell. Biol. 13:7652–7665; Levine *et al.*, 1993, Mol. Cell. Biol. 13:3494–3504: Ma *et al.*, 1996, J. Biol. Chem. 271:8144–8151). The zinc-finger protein tristetraprolin has been identified as another ARE-binding protein with destabilizing activity on TNFα, IL-3 and GM-CSF mRNAs (Stoecklin *et al.*, 2000, Mol. Cell. Biol. 20:3753–3763; Carballo *et al.*, 2000, Blood 95:1891–1899).

Since ARE-containing genes are clearly important in biological systems, including but not limited to a number of the early response genes that regulate cell proliferation and responses to exogenous agents, the identification of compounds that bind to one or more of the ARE clusters and potentially modulate the stability of the target RNA can potentially be of value as a therapeutic.

4.2. Detectably Labeled Target RNAs

Target nucleic acids, including but not limited to RNA and DNA, useful in the methods of the present invention have a label that is detectable via conventional spectroscopic means or radiographic means. Preferably, target nucleic acids are labeled with a covalently attached dye molecule. Useful dye-molecule labels include, but are not limited

to, fluorescent dyes, phosphorescent dyes, ultraviolet dyes, infrared dyes, and visible dyes. Preferably, the dye is a visible dye.

Useful labels in the present invention can include, but are not limited to, spectroscopic labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives such as fluorescein isothiocyanate (FITC) and Oregon Green™, rhodamine and derivatives (e.g., Texas red, tetramethylrhodimine isothiocynate (TRITC), bora-3a,4a-diaza-s-indacene (BODIPY®) and derivatives, *etc.*), digoxigenin, biotin, phycoerythrin, AMCA, CyDye™, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*), enzymes (e.g., horse radish peroxidase, alkaline phosphatase *etc.*), spectroscopic colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads, or nanoparticles – nanoclusters of inorganic ions with defined dimension from 0.1 to 1000 nm. The label may be coupled directly or indirectly to a component of the detection assay (*e.g.*, the detection reagent) according to methods well known in the art. A wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

In one embodiment, nucleic acids that are labeled at one or more specific locations are chemically synthesized using phosphoramidite or other solution or solid-phase methods. Detailed descriptions of the chemistry used to form polynucleotides by the phosphoramidite method are well known (*see, e.g.,* Caruthers *et al.,* U.S. Pat. Nos. 4,458,066 and 4,415,732; Caruthers *et al.*, 1982, Genetic Engineering 4:1-17; *Users Manual Model 392 and 394 Polynucleotide Synthesizers,* 1990, pages 6-1 through 6-22, Applied Biosystems, Part No. 901237; Ojwang, *et al.*, 1997, Biochemistry, 36:6033-6045). The phosphoramidite method of polynucleotide synthesis is the preferred method because of its efficient and rapid coupling and the stability of the starting materials. The synthesis is performed with the growing polynucleotide chain attached to a solid support, such that excess reagents, which are generally in the liquid phase, can be easily removed by washing, decanting, and/or filtration, thereby eliminating the need for purification steps between synthesis cycles.

The following briefly describes illustrative steps of a typical polynucleotide synthesis cycle using the phosphoramidite method. First, a solid support to which is attached a protected nucleoside monomer at its 3′ terminus is treated with acid, *e.g.*, trichloroacetic acid, to remove the 5’-hydroxyl protecting group, freeing the hydroxyl group for a subsequent coupling reaction. After the coupling reaction is completed an activated intermediate is formed by contacting the support-bound nucleoside with a protected nucleoside phosphoramidite monomer and a weak acid, *e.g.*, tetrazole. The weak acid

protonates the nitrogen atom of the phosphoramidite forming a reactive intermediate. Nucleoside addition is generally complete within 30 seconds. Next, a capping step is performed, which terminates any polynucleotide chains that did not undergo nucleoside addition. Capping is preferably performed using acetic anhydride and 1-methylimidazole. The phosphite group of the internucleotide linkage is then converted to the more stable phosphotriester by oxidation using iodine as the preferred oxidizing agent and water as the oxygen donor. After oxidation, the hydroxyl protecting group of the newly added nucleoside is removed with a protic acid, *e.g.*, trichloroacetic acid or dichloroacetic acid, and the cycle is repeated one or more times until chain elongation is complete. After synthesis, the polynucleotide chain is cleaved from the support using a base, *e.g.*, ammonium hydroxide or t-butyl amine. The cleavage reaction also removes any phosphate protecting groups, *e.g.*, cyanoethyl. Finally, the protecting groups on the exocyclic amines of the bases and any protecting groups on the dyes are removed by treating the polynucleotide solution in base at an elevated temperature, *e.g.*, at about 55°C. Preferably the various protecting groups are removed using ammonium hydroxide or t-butyl amine.

Any of the nucleoside phosphoramidite monomers can be labeled using standard phosphoramidite chemistry methods (Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002; Ojwang *et al.*, 1997, Biochemistry. 36:6033-6045 and references cited therein). Dye molecules useful for covalently coupling to phosphoramidites preferably comprise a primary hydroxyl group that is not part of the dye's chromophore. Illustrative dye molecules include, but are not limited to, disperse dye CAS 4439-31-0, disperse dye CAS 6054-58-6, disperse dye CAS 4392-69-2 (Sigma-Aldrich, St. Louis, MO), disperse red, and 1-pyrenebutanol (Molecular Probes, Eugene, OR). Other dyes useful for coupling to phosphoramidites will be apparent to those of skill in the art, such as fluoroscein, cy3, and cy5 fluorescent dyes, and may be purchased from, *e.g.*, Sigma-Aldrich, St. Louis, MO or Molecular Probes, Inc., Eugene, OR.

In another embodiment, dye-labeled target RNA molecules are synthesized enzymatically using *in vitro* transcription (Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002 and references cited therein). In this embodiment, a template DNA is denatured by heating to about 90°C and an oligonucleotide primer is annealed to the template DNA, for example by slow-cooling the mixture of the denatured template and the primer from about 90°C to room temperature. A mixture of ribonucleoside-5'-triphosphates capable of supporting template-directed enzymatic extension of the primed template (*e.g.*, a mixture including GTP, ATP, CTP, and UTP), including one or more dye-labeled ribonucleotides (Sigma-Aldrich, St. Louis, MO), is added to the primed template. Next, a polymerase

enzyme is added to the mixture under conditions where the polymerase enzyme is active, which are well-known to those skilled in the art. A labeled polynucleotide is formed by the incorporation of the labeled ribonucleotides during polymerase-mediated strand synthesis.

In yet another embodiment of the invention, nucleic acid molecules are end-labeled after their synthesis. Methods for labeling the 5'-end of an oligonucleotide include but are by no means limited to: (i) periodate oxidation of a 5'-to-5'-coupled ribonucleotide, followed by reaction with an amine-reactive label (Heller & Morisson, 1985, in *Rapid Detection and Identification of Infectious Agents,* D.T. Kingsbury and S. Falkow, eds., pp. 245-256, Academic Press); (ii) condensation of ethylenediamine with 5'-phosphorylated polynucleotide, followed by reaction with an amine reactive label (Morrison, European Patent Application 232 967); (iii) introduction of an aliphatic amine substituent using an aminohexyl phosphite reagent in solid-phase DNA synthesis, followed by reaction with an amine reactive label (Cardullo *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:8790-8794); and (iv) introduction of a thiophosphate group on the 5'-end of the nucleic acid, using phosphatase treatment followed by end-labeling with ATP- S and kinase, which reacts specifically and efficiently with maleimide-labeled fluorescent dyes (Czworkowski *et al.*, 1991, Biochem. 30:4821-4830).

A detectable label should not be incorporated into a target nucleic acid at the specific binding site at which test compounds are likely to bind, since the presence of a covalently attached label might interfere sterically or chemically with the binding of the test compounds at this site. Accordingly, if the region of the target nucleic acid that binds to a host cell factor is known, a detectable label is preferably incorporated into the nucleic acid molecule at one or more positions that are spatially or sequentially remote from the binding region.

After synthesis, the labeled target nucleic acid can be purified using standard techniques known to those skilled in the art (*see* Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002 and references cited therein). Depending on the length of the target nucleic acid and the method of its synthesis, such purification techniques include, but are not limited to, reverse-phase high-performance liquid chromatography ("reverse-phase HPLC"), fast performance liquid chromatography ("FPLC"), and gel purification. After purification, the target RNA is refolded into its native conformation, preferably by heating to approximately 85-95°C and slowly cooling to room temperature in a buffer, *e.g.*, a buffer comprising about 50 mM Tris-HCl, pH 8 and 100 mM NaCl.

In another embodiment, the target nucleic acid can also be radiolabeled. A radiolabel, such as, but not limited to, an isotope of phosphorus, sulfur, or hydrogen, may be

incorporated into a nucleotide, which is added either after or during the synthesis of the target nucleic acid. Methods for the synthesis and purification of radiolabeled nucleic acids are well known to one of skill in the art. *See, e.g.,* Sambrook *et al.*, 1989, in *Molecular Cloning: A Laboratory Manual*, pp 10.2-10.70, Cold Spring Harbor Laboratory Press, and the references cited therein, which are hereby incorporated by reference in their entireties.

In another embodiment, the target nucleic acid can be attached to an inorganic nanoparticle. A nanoparticle is a cluster of ions with controlled size from 0.1 to 1000 nm comprised of metals, metal oxides, or semiconductors including, but not limited to Ag_2S, ZnS, CdS, CdTe, Au, or TiO_2. Nanoparticles have unique optical, electronic and catalytic properties relative to bulk materials which can be adjusted according to the size of the particle. Methods for the attachment of nucleic acids are well know to one of skill in the art (see, *e.g.*, Niemeyer, 2001, Angew. Chem. Int. Ed. 40: 4129-4158, International Patent Publication WO/0218643, and the references cited therein, the disclosures of which are hereby incorporated by reference in their entireties).

4.3. Libraries of Small Molecules

Libraries screened using the methods of the present invention can comprise a variety of types of test compounds on solid supports. In all of the embodiments described below, all of the libraries can be synthesized on solid supports or the compounds of the library can be attached to solid supports by linkers.

In some embodiments, the test compounds are nucleic acid or peptide molecules. In a non-limiting example, peptide molecules can exist in a phage display library. In other embodiments, types of test compounds include, but are not limited to, peptide analogs including peptides comprising non-naturally occurring amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphoric acids and α-amino phosphoric acids, or amino acids having non-peptide linkages, nucleic acid analogs such as phosphorothioates and PNAs, hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose. Libraries of polypeptides or proteins can also be used.

In a preferred embodiment, the combinatorial libraries are small organic molecule libraries, such as, but not limited to, benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, and diazepindiones. In another embodiment, the combinatorial libraries comprise peptoids; random bio-oligomers; benzodiazepines; diversomers such as hydantoins, benzodiazepines and dipeptides;

vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; or carbohydrate libraries. Combinatorial libraries are themselves commercially available (see, *e.g.*, Advanced ChemTech Europe Ltd., Cambridgeshire, UK; ASINEX, Moscow Russia; BioFocus plc, Sittingbourne, UK; Bionet Research (A division of Key Organics Limited), Camelford, UK; ChemBridge Corporation, San Diego, California; ChemDiv Inc, San Diego, California.; ChemRx Advanced Technologies, South San Francisco, California; ComGenex Inc., Budapest, Hungary; Evotec OAI Ltd, Abingdon, UK; IF LAB Ltd., Kiev, Ukraine; Maybridge plc, Cornwall, UK; PharmaCore, Inc., North Carolina; SIDDCO Inc, Tucson, Arizona; TimTec Inc, Newark, Delaware; Tripos Receptor Research Ltd, Bude, UK; Toslab, Ekaterinburg, Russia).

In one embodiment, the combinatorial compound library for the methods of the present invention may be synthesized. There is a great interest in synthetic methods directed toward the creation of large collections of small organic compounds, or libraries, which could be screened for pharmacological, biological or other activity (Dolle, 2001, J. Comb. Chem. 3:477-517; Hall *et al.*, 2001, *ibid.* 3:125-150; Dolle, 2000, *ibid.* 2:383-433; Dolle, 1999, *ibid.* 1:235-282). The synthetic methods applied to create vast combinatorial libraries are performed in solution or in the solid phase, *i.e.*, on a solid support. Solid-phase synthesis makes it easier to conduct multi-step reactions and to drive reactions to completion with high yields because excess reagents can be easily added and washed away after each reaction step. Solid-phase combinatorial synthesis also tends to improve isolation, purification and screening. However, the more traditional solution phase chemistry supports a wider variety of organic reactions than solid-phase chemistry. Methods and strategies for the synthesis of combinatorial libraries can be found in *A Practical Guide to Combinatorial Chemistry*, A.W. Czarnik and S.H. Dewitt, eds., American Chemical Society, 1997; *The Combinatorial Index*, B.A. Bunin, Academic Press, 1998; *Organic Synthesis on Solid Phase*, F.Z. Dörwald, Wiley-VCH, 2000; and *Solid-Phase Organic Syntheses, Vol. 1*, A.W. Czarnik, ed., Wiley Interscience, 2001.

Combinatorial compound libraries of the present invention may be synthesized using apparatuses described in US Patent No. 6,358,479 to Frisina *et al.*, U.S. Patent No. 6,190,619 to Kilcoin *et al.*, US Patent No. 6,132,686 to Gallup *et al.*, US Patent No. 6,126,904 to Zuellig *et al.*, US Patent No. 6,074,613 to Harness *et al.*, US Patent No. 6,054,100 to Stanchfield *et al.*, and US Patent No. 5,746,982 to Saneii *et al.* which are hereby incorporated by reference in their entirety. These patents describe synthesis apparatuses

capable of holding a plurality of reaction vessels for parallel synthesis of multiple discrete compounds or for combinatorial libraries of compounds.

In one embodiment, the combinatorial compound library can be synthesized in solution. The method disclosed in U.S. Patent No. 6,194,612 to Boger *et al.,* which is hereby incorporated by reference in its entirety, features compounds useful as templates for solution phase synthesis of combinatorial libraries. The template is designed to permit reaction products to be easily purified from unreacted reactants using liquid/liquid or solid/liquid extractions. The compounds produced by combinatorial synthesis using the template will preferably be small organic molecules. Some compounds in the library may mimic the effects of non-peptides or peptides. In contrast to solid phase synthesize of combinatorial compound libraries, liquid phase synthesis does not require the use of specialized protocols for monitoring the individual steps of a multistep solid phase synthesis (Egner *et al.*, 1995, J.Org. Chem. 60:2652; Anderson *et al.*, 1995, J. Org. Chem. 60:2650; Fitch *et al.*, 1994, J. Org. Chem. 59:7955; Look *et al.*, 1994, J. Org. Chem. 49:7588; Metzger *et al.*, 1993, Angew. Chem., Int. Ed. Engl. 32:894; Youngquist *et al.*, 1994, Rapid Commun. Mass Spect. 8:77; Chu *et al.*, 1995, J. Am. Chem. Soc. 117:5419; Brummel *et al.*, 1994, Science 264:399; Stevanovic *et al.*, 1993, Bioorg. Med. Chem. Lett. 3:431).

Combinatorial compound libraries useful for the methods of the present invention can be synthesized on solid supports. In one embodiment, a split synthesis method, a protocol of separating and mixing solid supports during the synthesis, is used to synthesize a library of compounds on solid supports (*see* Lam *et al.*, 1997, Chem. Rev. 97:41-448; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926 and references cited therein). Each solid support in the final library has substantially one type of test compound attached to its surface. Other methods for synthesizing combinatorial libraries on solid supports, wherein one product is attached to each support, will be known to those of skill in the art (see, e.g., Nefzi et al., 1997, Chem. Rev. 97:449-472 and US Patent No. 6,087,186 to Cargill *et al.* which are hereby incorporated by reference in their entirety).

As used herein, the term "solid support" is not limited to a specific type of solid support. Rather a large number of supports are available and are known to one skilled in the art. Solid supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, polystyrene beads, doped polystyrene beads (as described by Fenniri *et al.*, 2000, J. Am. Chem. Soc. 123:8151-8152), alumina gels, and polysaccharides. A suitable solid support may be selected on the basis of desired end use and suitability for various synthetic protocols. For example, for peptide synthesis, a solid support can be a resin such as p-methylbenzhydrylamine (pMBHA) resin (Peptides International, Louisville, KY),

polystyrenes (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), including chloromethylpolystyrene, hydroxymethylpolystyrene and aminomethylpolystyrene, poly (dimethylacrylamide)-grafted styrene co-divinyl-benzene (*e.g.*, POLYHIPE resin, obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (*e.g.*, TENTAGEL or ARGOGEL, Bayer, Tubingen, Germany) polydimethylacrylamide resin (obtained from Milligen/Biosearch, California), or Sepharose (Pharmacia, Sweden). In another embodiment, the solid support can be a magnetic bead coated with streptavidin, such as Dynabeads Streptavidin (Dynal Biotech, Oslo, Norway).

In one embodiment, the solid phase support is suitable for *in vivo* use, *i.e.*, it can serve as a carrier or support for administration of the test compound to a patient (*e.g.*, TENTAGEL, Bayer, Tubingen, Germany). In a particular embodiment, the solid support is palatable and/or orally ingestable.

In some embodiments of the present invention, compounds can be attached to solid supports via linkers. Linkers can be integral and part of the solid support, or they may be nonintegral that are either synthesized on the solid support or attached thereto after synthesis. Linkers are useful not only for providing points of test compound attachment to the solid support, but also for allowing different groups of molecules to be cleaved from the solid support under different conditions, depending on the nature of the linker. For example, linkers can be, *inter alia*, electrophilically cleaved, nucleophilically cleaved, photocleavable, enzymatically cleaved, cleaved by metals, cleaved under reductive conditions or cleaved under oxidative conditions.

4.4. Library Screening

After a target nucleic acid, such as but not limited to RNA or DNA, is labeled and a test compound library is synthesized or purchased or both, the labeled target nucleic acid is used to screen the library to identify test compounds that bind to the nucleic acid. Screening comprises contacting a labeled target nucleic acid with an individual, or small group, of the components of the compound library. Preferably, the contacting occurs in an aqueous solution, and most preferably, under physiologic conditions. The aqueous solution preferably stabilizes the labeled target nucleic acid and prevents denaturation or degradation of the nucleic acid without interfering with binding of the test compounds. The aqueous solution can be similar to the solution in which a complex between the target RNA and its corresponding host cell factor is formed *in vitro*. For example, TK buffer, which is commonly used to form Tat protein-TAR RNA complexes *in vitro*, can be used in the

methods of the invention as an aqueous solution to screen a library of test compounds for TAR RNA binding compounds.

The methods of the present invention for screening a library of test compounds preferably comprise contacting a test compound with a target nucleic acid in the presence of an aqueous solution, the aqueous solution comprising a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions. The aqueous solution optionally further comprises non-specific nucleic acids, such as, but not limited to, DNA; yeast tRNA; salmon sperm DNA; homoribopolymers such as, but not limited to, poly IC, polyA, polyU, and polyC; and non-specific RNA. The non-specific RNA may be an unlabeled target nucleic acid having a mutation at the binding site, which renders the unlabeled nucleic acid incapable of interacting with a test compound at that site. For example, if dye-labeled TAR RNA is used to screen a library, unlabeled TAR RNA having a mutation in the uracil 23/cytosine 24 bulge region may also be present in the aqueous solution. Without being bound by any theory, the addition of unlabeled RNA that is essentially identical to the dye-labeled target RNA except for a mutation at the binding site might minimize interactions of other regions of the dye-labeled target RNA with test compounds or with the solid support and prevent false positive results.

The solution further comprises a buffer, a combination of salts, and optionally, a detergent or a surfactant. The pH of the solution typically ranges from about 5 to about 8, preferably from about 6 to about 8, most preferably from about 6.5 to about 8. A variety of buffers may be used to achieve the desired pH. Suitable buffers include, but are not limited to, Tris, Mes, Bis-Tris, Ada, Aces, Pipes, Mopso, Bis-Tris propane, Bes, Mops, Tes, Hepes, Dipso, Mobs, Tapso, Trizma, Heppso, Popso, TEA, Epps, Tricine, Gly-Gly, Bicine, and sodium-potassium phosphate. The buffering agent comprises from about 10 mM to about 100 mM, preferably from about 25 mM to about 75 mM, most preferably from about 40 mM to about 60 mM buffering agent. The pH of the aqeuous solution can be optimized for different screening reactions, depending on the target RNA used and the types of test compounds in the library, and therefore, the type and amount of the buffer used in the solution can vary from screen to screen. In a preferred embodiment, the aqueous solution has a pH of about 7.4, which can be achieved using about 50 mM Tris buffer.

In addition to an appropriate buffer, the aqueous solution further comprises a combination of salts, from about 0 mM to about 100 mM KCl, from about 0 mM to about 1 M NaCl, and from about 0 mM to about 200 mM $MgCl_2$. In a preferred embodiment, the combination of salts is about 100 mM KCl, 500 mM NaCl, and 10 mM $MgCl_2$. Without being bound by any theory, Applicant has found that a combination of KCl, NaCl, and $MgCl_2$

stabilizes the target RNA such that most of the RNA is not denatured or digested over the course of the screening reaction. The optional concentration of each salt used in the aqueous solution is dependent on the particular target RNA used and can be determined using routine experimentation.

The solution optionally comprises from about 0.01% to about 0.5% (w/v) of a detergent or a surfactant. Without being bound by any theory, a small amount of detergent or surfactant in the solution might reduce non-specific binding of the target RNA to the solid support and control aggregation and increase stability of target RNA molecules. Typical detergents useful in the methods of the present invention include, but are not limited to, anionic detergents, such as salts of deoxycholic acid, 1-heptanesulfonic acid, N-laurylsarcosine, lauryl sulfate, 1-octane sulfonic acid and taurocholic acid; cationic detergents such as benzalkonium chloride, cetylpyridinium, methylbenzethonium chloride, and decamethonium bromide; zwitterionic detergents such as CHAPS, CHAPSO, alkyl betaines, alkyl amidoalkyl betaines, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and phosphatidylcholine; and non-ionic detergents such as n-decyl a-D-glucopyranoside, n-decyl ß-D-maltopyranoside, n-dodecyl ß-D-maltoside, n-octyl ß-D-glucopyranoside, sorbitan esters, n-tetradecyl ß-D-maltoside, octylphenoxy polyethoxyethanol (Nonidet P-40), nonylphenoxypolyethoxyethanol (NP-40), and tritons. Preferably, the detergent, if present, is a nonionic detergent. Typical surfactants useful in the methods of the present invention include, but are not limited to, ammonium lauryl sulfate, polyethylene glycols, butyl glucoside, decyl glucoside, Polysorbate 80, lauric acid, myristic acid, palmitic acid, potassium palmitate, undecanoic acid, lauryl betaine, and lauryl alcohol. More preferably, the detergent, if present, is Triton X-100 and present in an amount of about 0.1% (w/v).

Non-specific binding of a labeled target nucleic acid to test compounds can be further minimized by treating the binding reaction with one or more blocking agents. In one embodiment, the binding reactions are treated with a blocking agent, *e.g.*, bovine serum albumin ("BSA"), before contacting with to the labeled target nucleic acid. In another embodiment, the binding reactions are treated sequentially with at least two different blocking agents. This blocking step is preferably performed at room temperature for from about 0.5 to about 3 hours. In a subsequent step, the reaction mixture is further treated with unlabeled RNA having a mutation at the binding site. This blocking step is preferably performed at about 4°C for from about 12 hours to about 36 hours before addition of the dye-labeled target RNA. Preferably, the solution used in the one or more blocking steps is substantially similar to the aqueous solution used to screen the library with the dye-labeled target RNA, *e.g.*, in pH and salt concentration.

Once contacted, the mixture of labeled target nucleic acid and the test compound is preferably maintained at 4°C for from about 1 day to about 5 days, preferably from about 2 days to about 3 days with constant agitation. To identify the reactions in which binding to the labeled target nucleic acid occurred, after the incubation period, bound from free compounds are determined using any of the methods disclosed in Section 4.5 *infra*.

4.5. Separation Methods for Screening Test Compounds

After the labeled target RNA is contacted with the library of test compounds immobilized on beads, the beads must then be separated from the unbound target RNA in the liquid phase. This can be accomplished by any number of physical means; *e.g.*, sedimentation, centrifugation. Thereafter, a number of methods can be used to separate the library beads that are complexed with the labeled target RNA from uncomplexed beads in order to isolate the test compound on the bead. Alternatively, mass spectroscopy and NMR spectroscopy can be used to simultaneously identify and separate beads complexed to the labeled target RNA from uncomplexed beads.

4.5.1. Flow Cytometry

In a preferred embodiment, the complexed and non-complexed target nucleic acids are separated by flow cytometry methods. Flow cytometers for sorting and examining biological cells are well known in the art; this technology can be applied to separate the labeled library beads from unlabeled beads. Known flow cytometers are described, for example, in U.S. Patent Nos. 4,347,935; 5,464,581; 5,483,469; 5,602,039; 5,643,796; and 6,211,477; the entire contents of which are incorporated by reference herein. Other known flow cytometers are the FACS Vantage™ system manufactured by Becton Dickinson and Company, and the COPAS™ system manufactured by Union Biometrica.

A flow cytometer typically includes a sample reservoir for receiving a biological sample. The biological sample contains particles (hereinafter referred to as "beads") that are to be analyzed and sorted by the flow cytometer. Beads are transported from the sample reservoir at high speed (>100beads/second) to a flow cell in a stream of liquid "sheath" fluid. High-frequency vibrations of a nozzle that directs the stream to the flow cell causes the stream to partition and form ordered droplets, with each droplet containing a single bead. Physical properties of beads can be measured as they intersect a laser beam within the cytometer flow cell. As beads move one by one through the interrogation point, they cause the laser light to scatter and fluorescent molecules on the labeled beads (*i.e.*, beads complexed with labeled target RNA) become excited.

Alternatively, if the target nucleic acid is labeled with an inorganic nanoparticle, the beads complexed with bound target nucleic acid can be distinguished not only by unique fluorescent properties but also on the basis of spectrometric properties (*e.g.* including but not limited to increased optical density due to the reduction of Ag^+ ions in the presence of gold nanoparticles (see, *e.g.*, Taton *et al.* Science 2000, 289: 1757-1760)).

An appropriate detection system consisting of photomultiplier tubes, photodiodes or other devices for measuring light are focused onto the interrogation point where the properties are measured. In so doing, information regarding particle size (light scatter) and complex formation (fluorescence intensity) is obtained. Particles with the desired physical properties are then sorted by a variety of physical means. In one embodiment, the beads are sorted by an electrostatic method. To sort beads by an electrostatic method, the droplets containing the beads with the desired physical properties are electrically charged and deflected from the trajectory of uncharged droplets as they pass through an electrostatic field formed by two deflection plates held constant at a high electrical potential difference. In another embodiment, the beads are sorted by an air-diverting method. To sort beads by an air-diverting method, the droplets containing the beads with the desired physical properties are deflected from their trajectory by a focused stream of forced air. Both of these embodiments cause the trajectory of beads with the desired physical properties to become changed, thereby sorting them from other beads. Accordingly, the beads complexed to the labeled target RNA can be collected in an appropriate collecting vessel.

Thus, in one embodiment of the present invention, the complexed and non-complexed target nucleic acids are separated by flow cytometry methods. In a preferred embodiment, the target nucleic acid is labeled with a fluorescent label and the complexed and non-complexed target nucleic acids are separated by fluorescence activated cell sorting ("FACS"). Such methods are well known to one of skill in the art.

4.5.2. <u>Affinity Chromatography</u>

In another embodiment of the invention, the target RNA can be labeled with biotin, an antigen, or a ligand. Library beads complexed to the target RNA can be separated from uncomplexed beads using affinity techniques designed to capture the labeled moiety on the target RNA. For example, a solid support, such as but not limited to, a column or a well in a microwell plate coated with avidin/streptavidin, an antibody to the antigen, or a receptor for the ligand can be used to capture or immobilize the labeled beads. Complexed RNA may or may not be irreversibly bound to the bead by a further transformation between the bound

RNA and an additional moiety on the surface of the bead. Such linking methods include, but are not limited to: photochemical crosslinking between RNA and bead-bound molecules such as psoralen, thymidine or uridine derivates either present as monomers, oligomers, or as a partially complementary sequence; or chemical ligation by disulfide exchange, nitrogen mustards, bond formation between an electrophile and a nucleophile, or alkylating reagents. See, *e.g.*, International Patent Publication WO/0146461, the contents of which are hereby incorporated by reference. The unbound library beads can be removed after the binding reaction by washing the solid phase. If the RNA is irreversibly bound to the bead, test compounds can be isolated from the bead following destruction of the bound RNA by preferably, but not limited to, enzymatic or chemical (*e.g.*, alkaline hydrolysis) degradation. The library beads bound to the solid phase can then be eluted with any solution that disrupts the binding between the labeled target RNA and the solid phase. Such solutions include high salt solutions, low pH solutions, detergents, and chaotropic denaturants, and are well known to one of skill in the art. In another embodiment, the test compounds can be eluted from the solid phase by heat.

In one embodiment, the library of test compounds can be prepared on magnetic beads, such as Dynabeads Streptavidin (Dynal Biotech, Oslo, Norway). The magnetic bead library can then be mixed with the labeled target RNA under conditions that allow binding to occur. The separation of the beads from unbound target RNA in the liquid phase can be accomplished using a magnet. After removal of the magnetic field, the bead complexed to the labeled RNA may be separated from uncomplexed library beads via the label used on the target RNA; *e.g.*, biotinylated target RNA can be captured by avidin/streptavidin; target RNA labeled with antigen can be captured by the appropriate antibody; target RNA labeled with ligand can be captured using the appropriate immobilized receptor. The captured library bead can then be eluted with any solution that disrupts the binding between the labeled target RNA and the immobilized surface. Such solutions include high salt solutions, low pH solutions, detergents, and chaotropic denaturants, and are well known to one of skill in the art. Complexed RNA may or may not be irreversibly bound to the bead by a further transformation between the bound RNA and an additional moiety on the surface of the bead. Such linking methods include, but are not limited to: photochemical crosslinking between RNA and bead-bound molecules such as psoralen, thymidine or uridine derivates either present as monomers, oligomers, or as a partially complementary sequence; or chemical ligation by disulfide exchange, nitrogen mustards, bond formation between an electrophile and a nucleophile, or alkylating reagents. See, *e.g.*, International Patent Publication WO/0146461, the contents of which are hereby incorporated by reference. If the

RNA is irreversibly bound to the bead, test compounds can be isolated from the bead following destruction of the bound RNA by enzymatic degradation including, but not limited to, ribonucleases A, U_2, CL_3, T_1, Phy M, *B. cereus* or chemical degradation including, but not limited to, piperidine-promoted backbone cleavage of abasic sites (following treatment with sodium hydroxide, hydrazine, piperidine formate, or dimethyl sulfate), or metal-assisted (*e.g.* nickel(II), cobalt(II), or iron(II)) oxidative cleavage.

In another embodiment, the preselected target RNA can be labeled with a heavy metal tag and incubated with the library beads to allow binding of the test compounds to the target RNA. The separation of the labeled beads from unlabeled beads can be accomplished using a magnetic field. After removal of the magnetic field, the test compound can be eluted with any solution that disrupts the binding between the preselected target RNA and the test compound. Such solutions include high salt solutions, low pH solutions, detergents, and chaotropic denaturants, and are well known to one of skill in the art. In another embodiment, the test compounds can be eluted from the solid phase by heat.

4.5.3. Manual Batch

In one embodiment, a manual "batch" mode is used for separating complexed beads. To explore a bead-based library within a reasonable time period, the primary screens should be operated with sufficient throughput. To do this, the target nucleic acid is labeled with a dye and then incubated with the combinatorial library. An advantage of such an assay is the fast identification of active library beads by color change. In the lower concentrations of the dye-labeled target molecule, only those library beads that bind the target molecules most tightly are detected because of higher local concentration of the dye. When washed and plated into a liquid monolayer, colored beads are easily separated from non-colored beads with the aid of a dissecting microscope. One of the problems associated with this method could be the interaction between the red dye and library substrates. Control experiments using the dye alone and dye attached to mutant RNA sequences with the libraries are performed to eliminate this possibility.

4.5.4. Suspension of Beads in Electric Fields

In another embodiment of the invention, library beads bound to the target RNA can be separated from unbound beads on the basis of the altered charge properties due to RNA binding. In a preferred embodiment of this technique, beads are separated from unbound nucleic acid and suspended, preferably but not only, in the presence of an electric field where the bound RNA causes the beads bound to the target RNA to migrate toward the

anode, or positive, end of the field.

Beads can be preferentially suspended in solution as a colloidal suspension with the aid of detergents or surfactants. Typical detergents useful in the methods of the present invention include, but are not limited to, anionic detergents, such as salts of deoxycholic acid, 1-heptanesulfonic acid, N-laurylsarcosine, lauryl sulfate, 1-octane sulfonic acid, carboxymethylcellulose, carrageenan, and taurocholic acid; cationic detergents such as benzalkonium chloride, cetylpyridinium, methylbenzethonium chloride, and decamethonium bromide; zwitterionic detergents such as CHAPS, CHAPSO, alkyl betaines, alky amidoalkyl betaines, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and phosphatidylcholine; and non-ionic detergents such as n-decyl α-D-glucopyranoside, n-decyl-D-maltopyranoside, n-dodecyl -D-maltoside, n-octyl -D-glucopyranoside, sorbitan esters, n-tetradecyl -D-maltoside and tritons. Preferably, the detergent, if present, is a nonionic detergent. Typical surfactants useful in the methods of the present invention include, but are not limited to, ammonium lauryl sulfate, polyethylene glycols, butyl glucoside, decyl glucoside, Polysorbate 80, lauric acid, myristic acid, palmitic acid, potassium palmitate, undecanoic acid, lauryl betaine, and lauryl alcohol.

Complexed RNA may or may not be irreversibly bound to the bead by a further transformation between the bound RNA and an additional moiety on the surface of the bead. Such linking methods include, but are not limited to: photochemical crosslinking between RNA and bead-bound molecules such as psoralen, thymidine or uridine derivates either present as monomers, oligomers, or as a partially complementary sequence; or chemical ligation by disulfide exchange, nitrogen mustards, bond formation between an electrophile and a nucleophile, or alkylating reagents.

If the RNA is irreversibly bound to the bead, test compounds can be isolated from the bead following destruction of the bound RNA by enzymatic degradation including, but not limited to, ribonucleases A, U_2, CL_3, T_1, Phy M, *B. cereus* or chemical degradation including, but not limited to, piperidine-promoted backbone cleavage of abasic sites (following treatment with sodium hydroxide, hydrazine, piperidine formate, or dimethyl sulfate), or metal-assisted (*e.g.* nickel(II), cobalt(II), or iron(II)) oxidative cleavage.

4.5.5. Microwave

In another embodiment, the complexed beads are separated from uncomplexed beads by microwave. For example, as described in U.S. Patent Nos. 6,340,568; 6,338,968; and 6,287,874 to Hefti, the disclosures of which are hereby incorporated by reference, a system which is sensitive to the unique dielectric properties of

molecules and binding complexes, such as hybridization complexes formed between a nucleic acid probe and a nucleic acid target, molecular binding events, and protein/ligand complexes, can be used to analyze nucleic acids. In this system, the different hybridization complexes can be directly distinguished without the use of labels. The method involves contacting a nucleic acid probe that is electromagnetically coupled to a portion of a signal path with a sample containing a target nucleic acid. The portion of the signal path to which the nucleic acid probe is coupled typically is a continuous transmission line. A response signal is detected for a hybridization complex formed between the nucleic acid probe and the nucleic acid target. Detection may involve propagating a test signal along the signal path and then detecting a response signal formed through modulation of the test signal by the hybridization complex.

4.6. <u>Methods for Identifying Test Compounds</u>

If the library is a peptide or nucleic acid library, the sequence of the test compound on the isolated bead can be determined by direct sequencing of the peptide or nucleic acid. Such methods are well known to one of skill in the art.

4.6.1. <u>Mass Spectrometry</u>

Mass spectrometry (*e.g.,* electrospray ionization ("ESI") and matrix-assisted laser desorption-ionization ("MALDI"), Fourier-transform ion cyclotron resonance ("FT-ICR")) can be used both for high-throughput screening of test compounds that bind to a target RNA and elucidating the structure of the test compound on the isolated bead.

MALDI uses a pulsed laser for desorption of the ions and a time-of-flight analyzer, and has been used for the detection of noncovalent tRNA:amino-acyl-tRNA synthetase complexes (Gruic-Sovulj *et al.*, 1997, J. Biol. Chem. 272:32084-32091). However, covalent cross-linking between the target nucleic acid and the test compound is required for detection, since a non-covalently bound complex may dissociate during the MALDI process.

ESI mass spectrometry ("ESI-MS") has been of greater utility for studying non-covalent molecular interactions because, unlike the MALDI process, ESI-MS generates molecular ions with little to no fragmentation (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356). ESI-MS has been used to study the complexes formed by HIV Tat peptide and protein with the TAR RNA (Sannes-Lowery *et al.*, 1997, Anal. Chem. 69:5130-5135).

Fourier-transform ion cyclotron resonance ("FT-ICR") mass spectrometry provides high-resolution spectra, isotope-resolved precursor ion selection, and accurate mass

assignments (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356). FT-ICR has been used to study the interaction of aminoglycoside antibiotics with cognate and non-cognate RNAs (Hofstadler *et al.*, 1999, Anal. Chem. 71:3436-3440; Griffey *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96:10129-10133). As true for all of the mass spectrometry methods discussed herein, FT-ICR does not require labeling of the target RNA or a test compound.

An advantage of mass spectroscopy is not only the elucidation of the structure of the test compound, but also the determination of the structure of the test compound bound to the preselected target RNA. Such information can enable the discovery of a consensus structure of a test compound that specifically binds to a preselected target RNA.

In a preferred embodiment, the structure of the test compound is determined by time of flight mass spectroscopy ("TOF-MS"). In time of flight methods of mass spectrometry, charged (ionized) molecules are produced in a vacuum and accelerated by an electric field into a time of flight tube or drift tube. The velocity to which the molecules may be accelerated is proportional to the accelerating potential, proportional to the charge of the molecule, and inversely proportional to the square of the mass of the molecule. The charged molecules travel, *i.e.*, "drift" down the TOF tube to a detector. The time taken for the molecules to travel down the tube may be interpreted as a measure of their molecular weight. Time-of-flight mass spectrometers have been developed for all of the major ionization techniques such as, but limited to, electron impact ("EI"), infrared laser desorption ("IRLD"), plasma desorption ("PD"), fast atom bombardment ("FAB"), secondary ion mass spectrometry ("SIMS"), matrix-assisted laser desorption/ionization ("MALDI"), and electrospray ionization ("ESI").

4.6.2. NMR Spectroscopy

NMR spectroscopy can be used for elucidating the structure of the test compound on the isolated bead. NMR spectroscopy is a technique for identifying binding sites in target nucleic acids by qualitatively determining changes in chemical shift, specifically from distances measured using relaxation effects. Examples of NMR that can be used for the invention include, but are not limited to, one-dimentional NMR, two-dimentional NMR, correlation spectroscopy ("COSY"), and nuclear Overhauser effect ("NOE") spectroscopy. Such methods of structure determination of test compounds are well known to one of skill in the art.

Similar to mass spectroscopy, an advantage of NMR is the not only the elucidation of the structure of the test compound, but also the determination of the structure of the test compound bound to the preselected target RNA. Such information can enable the

discovery of a consensus structure of a test compound that specifically binds to a preselected target RNA.

4.6.3. Edman Degradation

In an embodiment wherein the library is a peptide library or a derivative thereof, Edman degradation can be used to determine the structure of the test compound. In one embodiment, a modified Edman degradation process is used to obtain compositional tags for proteins, which is described in U.S. Patent No. 6,277,644 to Farnsworth *et al.*, which is hereby incorporated by reference in its entirety. The Edman degradation chemistry is separated from amino acid analysis, circumventing the serial requirement of the conventional Edman process. Multiple cycles of coupling and cleavage are performed prior to extraction and compositional analysis of amino acids. The amino acid composition information is then used to search a database of known protein or DNA sequences to identify the sample protein. An apparatus for performing this method comprises a sample holder for holding the sample, a coupling agent supplier for supplying at least one coupling agent, a cleavage agent supplier for supplying a cleavage agent, a controller for directing the sequential supply of the coupling agents, cleavage agents, and other reagents necessary for performing the modified Edman degradation reactions, and an analyzer for analyzing amino acids.

In another embodiment, the method can be automated as described in U.S. Patent No. 5,565,171 to Dovichi *et al.*, which is hereby incorporated by reference in its entirety. The apparatus includes a continuous capillary connected between two valves that control fluid flow in the capillary. One part of the capillary forms a reaction chamber where the sample may be immobilized for subsequent reaction with reagents supplied through the valves. Another part of the capillary passes through or terminates in the detector portion of an analyzer such as an electrophoresis apparatus, liquid chromatographic apparatus or mass spectrometer. The apparatus may form a peptide or protein sequencer for carrying out the Edman degradation reaction and analyzing the reaction product produced by the reaction. The protein or peptide sequencer includes a reaction chamber for carrying out coupling and cleavage on a peptide or protein to produce derivatized amino acid residue, a conversion chamber for carrying out conversion and producing a converted amino acid residue and an analyzer for identifying the converted amino acid residue. The reaction chamber may be contained within one arm of a capillary and the conversion chamber is located in another arm of the capillary. An electrophoresis length of capillary is directly capillary coupled to the conversion chamber to allow electrophoresis separation of the converted amino acid residue

as it leaves the conversion chamber. Identification of the converted amino acid residue takes place at one end of the electrophoresis length of the capillary.

4.6.4. Vibrational Spectroscopy

Vibrational spectroscopy (*e.g.* infrared (IR) spectroscopy or Raman spectroscopy) can be used for elucidating the structure of the test compound on the isolated bead.

Infrared spectroscopy measures the frequencies of infrared light (wavelengths from 100 to 10,000 nm) absorbed by the test compound as a result of excitation of vibrational modes according to quantum mechanical selection rules which require that absorption of light cause a change in the electric dipole moment of the molecule. The infrared spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

Infrared spectra can be measured in a scanning mode by measuring the absorption of individual frequencies of light, produced by a grating which separates frequencies from a mixed-frequency infrared light source, by the test compound relative to a standard intensity (double-beam instrument) or pre-measured ('blank') intensity (single-beam instrument). In a preferred embodiment, infrared spectra are measured in a pulsed mode (FT-IR) where a mixed beam, produced by an interferometer, of all infrared light frequencies is passed through or reflected off the test compound. The resulting interferogram, which may or may not be added with the resulting interferograms from subsequent pulses to increase the signal strength while averaging random noise in the electronic signal, is mathematically transformed into a spectrum using Fourier Transform or Fast Fourier Transform algorithms.

Raman spectroscopy measures the difference in frequency due to absorption of infrared frequencies of scattered visible or ultraviolet light relative to the incident beam. The incident monochromatic light beam, usually a single laser frequency, is not truly absorbed by the test compound but interacts with the electric field transiently. Most of the light scattered off the sample with be unchanged (Rayleigh scattering) but a portion of the scatter light will have frequencies that are the sum or difference of the incident and molecular vibrational frequencies. The selection rules for Raman (inelastic) scattering require a change in polarizability of the molecule. While some vibrational transitions are observable in both infrared and Raman spectrometry, must are observable only with one or the other technique. The Raman spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

Raman spectra are measured by submitting monochromatic light to the sample, either passed through or preferably reflected off, filtering the Rayleigh scattered light, and detecting the frequency of the Raman scattered light. An improved Raman spectrometer is described in US Patent No. 5,786,893 to Fink *et al.*, which is hereby incorporated by reference.

Vibrational microscopy can be measured in a spatially resolved fashion to address single beads by integration of a visible microscope and spectrometer. A microscopic infrared spectrometer is described in U.S. Patent No. 5,581,085 to Reffner *et al.*, which is hereby incorporated by reference in its entirety. An instrument that simultaneously performs a microscopic infrared and microscopic Raman analysis on a sample is described in U.S. Patent No. 5,841,139 to Sostek *et al.*, which is hereby incorporated by reference in its entirety.

In one embodiment of the method, test compounds are synthesized on polystyrene beads doped with chemically modified styrene monomers such that each resulting bead has a characteristic pattern of absorption lines in the vibrational (IR or Raman) spectrum, by methods including but not limited to those described by Fenniri *et al.*, 2000, J. Am. Chem. Soc. 123:8151-8152. Using methods of split-pool synthesis familiar to one of skill in the art, the library of compounds is prepared so that the spectroscopic pattern of the bead identifies one of the components of the test compound on the bead. Beads that have been separated according to their ability to bind target RNA can be identified by their vibrational spectrum. In one embodiment of the method, appropriate sorting and binning of the beads during synthesis then allows identification of one or more further components of the test compound on any one bead. In another embodiment of the method, partial identification of the compound on a bead is possible through use of the spectroscopic pattern of the bead with or without the aid of further sorting during synthesis, followed by partial resynthesis of the possible compounds aided by doped beads and appropriate sorting during synthesis.

In another embodiment, the IR or Raman spectra of test compounds are examined while the compound is still on a bead, preferably, or after cleavage from bead, using methods including but not limited to photochemical, acid, or heat treatment. The test compound can be identified by comparison of the IR or Raman spectral pattern to spectra previously acquired for each test compound in the combinatorial library.

4.7. Secondary Biological Screens

The test compounds identified in the binding assay (for convenience referred to herein as a "lead" compound) can be tested for biological activity using host cells containing or engineered to contain the target RNA element coupled to a functional readout system. For example, the lead compound can be tested in a host cell engineered to contain the target RNA element controlling the expression of a reporter gene. In this example, the lead compounds are assayed in the presence or absence of the target RNA. Alternatively, a phenotypic or physiological readout can be used to assess activity of the target RNA in the presence and absence of the lead compound.

In one embodiment, the lead compound can be tested in a host cell engineered to contain the target RNA element controlling the expression of a reporter gene, such as, but not limited to, β-galactosidase, green fluorescent protein, red fluorescent protein, luciferase, chloramphenicol acetyltransferase, alkaline phosphatase, and β-lactamase. In a preferred embodiment, a cDNA encoding the target element is fused upstream to a reporter gene wherein translation of the reporter gene is repressed upon binding of the lead compound to the target RNA. In other words, the steric hindrance caused by the binding of the lead compound to the target RNA repressed the translation of the reporter gene. This method, termed the translational repression assay procedure ("TRAP") has been demonstrated in *E. coli* and *S. cerevisiae* (Jain & Belasco, 1996, Cell 87(1):115-25; Huang & Schreiber, 1997, Proc. Natl. Acad. Sci. USA 94:13396-13401).

In another embodiment, a phenotypic or physiological readout can be used to assess activity of the target RNA in the presence and absence of the lead compound. For example, the target RNA may be overexpressed in a cell in which the target RNA is endogenously expressed. Where the target RNA controls expression of a gene product involved in cell growth or viability, the *in vivo* effect of the lead compound can be assayed by measuring the cell growth or viability of the target cell. Alternatively, a reporter gene can also be fused downstream of the target RNA sequence and the effect of the lead compound on reporter gene expression can be assayed.

Alternatively, the lead compounds identified in the binding assay can be tested for biological activity using animal models for a disease, condition, or syndrome of interest. These include animals engineered to contain the target RNA element coupled to a functional readout system, such as a transgenic mouse. Animal model systems can also be used to demonstrate safety and efficacy.

Compounds displaying the desired biological activity can be considered to be lead compounds, and will be used in the design of congeners or analogs possessing useful

pharmacological activity and physiological profiles. Following the identification of a lead compound, molecular modeling techniques can be employed, which have proven to be useful in conjunction with synthetic efforts, to design variants of the lead that can be more effective. These applications may include, but are not limited to, Pharmacophore Modeling (*cf.* Lamothe, *et al.* 1997, J. Med. Chem. 40: 3542; Mottola *et al.* 1996, J. Med. Chem. 39: 285; Beusen *et al.* 1995, Biopolymers 36: 181; P. Fossa *et al.* 1998, Comput. Aided Mol. Des. 12: 361), QSAR development (*cf.* Siddiqui *et al.* 1999, J. Med. Chem. 42: 4122; Barreca *et al.* 1999 Bioorg. Med. Chem. 7: 2283; Kroemer *et al.* 1995, J. Med. Chem. 38: 4917; Schaal *et al.* 2001, J. Med. Chem. 44: 155; Buolamwini & Assefa 2002, J. Mol. Chem. 45: 84), Virtual docking and screening/scoring (*cf.* Anzini *et al.* 2001, J. Med. Chem. 44: 1134; Faaland *et al.* 2000, Biochem. Cell. Biol. 78: 415; Silvestri *et al.* 2000, Bioorg. Med. Chem. 8: 2305; J. Lee *et al.* 2001, Bioorg. Med. Chem. 9: 19), and Structure Prediction using RNA structural programs including, but not limited to mFold (as described by Zuker *et al.* Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology pp. 11-43, J. Barciszewski & B.F.C. Clark, eds. (NATO ASI Series, Kluwer Academic Publishers, 1999) and Mathews *et al.* 1999 J. Mol. Biol. 288: 911-940); RNAmotif (Macke *et al.* 2001, Nucleic Acids Res. 29: 4724-4735; and the Vienna RNA package (Hofacker *et al.* 1994, Monatsh. Chem. 125: 167-188).

Further examples of the application of such techniques can be found in several review articles, such as Rotivinen *et al.*, 1988, Acta Pharmaceutical Fennica 97:159-166; Ripka, 1998, New Scientist 54-57; McKinaly & Rossmann, 1989, Annu. Rev. Pharmacol. Toxiciol. 29:111-122; Perry & Davies, QSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis & Dean, 1989, Proc. R. Soc. Lond. 236:125-140 and 141-162; Askew et al., 1989, J. Am. Chem. Soc. 111:1082-1090. Molecular modeling tools employed may include those from Tripos, Inc., St. Louis, Missouri (*e.g.*, Sybyl/UNITY, CONCORD, DiverseSolutions), Accelerys, San Diego, California (*e.g.*, Catalyst, Wisconsin Package {BLAST, etc.}), Schrodinger, Portland, Oregon (*e.g.*, QikProp, QikFit, Jaguar) or other such vendors as BioDesign, Inc. (Pasadena, California), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario, Canada), and may include privately designed and/or "academic" software (*e.g.* RNAMotif, mFOLD). These application suites and programs include tools for the atomistic construction and analysis of structural models for drug-like molecules, proteins, and DNA or RNA and their potential interactions. They also provide for the calculation of important physical properties, such as solubility estimates, permeability metrics, and empirical measures of molecular "druggability" (*e.g.*, Lipinski "Rule of 5" as described by Lipinski *et al.* 1997, Adv. Drug

Delivery Rev. 23: 3-25). Most importantly, they provide appropriate metrics and statistical modeling power (such as the patented CoMFA technology in Sybyl as described in US Patents 6,240,374 and 6,185,506) to develop Quantitative Structural Activity Relationships (QSARs) which are used to guide the synthesis of more efficacious clinical development candidates while improving desirable physical properties, as determined by results from the aforementioned secondary screening protocols.

4.8. Use of Identified Compounds That Bind RNA to Treat/Prevent Disease

Biologically active compounds identified using the methods of the invention or a pharmaceutically acceptable salt thereof can be administered to a patient, preferably a mammal, more preferably a human, suffering from a disease whose progression is associated with a target RNA:host cell factor interaction *in vivo*. In certain embodiments, such compounds or a pharmaceutically acceptable salt thereof is administered to a patient, preferably a mammal, more preferably a human, as a preventative measure against a disease associated with an RNA:host cell factor interaction *in vivo*.

In one embodiment, "treatment" or "treating" refers to an amelioration of a disease, or at least one discernible symptom thereof. In another embodiment, "treatment" or "treating" refers to an amelioration of at least one measurable physical parameter, not necessarily discernible by the patient. In yet another embodiment, "treatment" or "treating" refers to inhibiting the progression of a disease, either physically, *e.g.*, stabilization of a discernible symptom, physiologically, *e.g.*, stabilization of a physical parameter, or both. In yet another embodiment, "treatment" or "treating" refers to delaying the onset of a disease.

In certain embodiments, the compound or a pharmaceutically acceptable salt thereof is administered to a patient, preferably a mammal, more preferably a human, as a preventative measure against a disease associated with an RNA:host cell factor interaction *in vivo*. As used herein, "prevention" or "preventing" refers to a reduction of the risk of acquiring a disease. In one embodiment, the compound or a pharmaceutically acceptable salt thereof is administered as a preventative measure to a patient. According to this embodiment, the patient can have a genetic predisposition to a disease, such as a family history of the disease, or a non-genetic predisposition to the disease. Accordingly, the compound and pharmaceutically acceptable salts thereof can be used for the treatment of one manifestation of a disease and prevention of another.

When administered to a patient, the compound or a pharmaceutically acceptable salt thereof is preferably administered as component of a composition that optionally comprises a pharmaceutically acceptable vehicle. The composition can be

administered orally, or by any other convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal, and intestinal mucosa, *etc.*) and may be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*, and can be used to administer the compound and pharmaceutically acceptable salts thereof.

Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. The mode of administration is left to the discretion of the practitioner. In most instances, administration will result in the release of the compound or a pharmaceutically acceptable salt thereof into the bloodstream.

In specific embodiments, it may be desirable to administer the compound or a pharmaceutically acceptable salt thereof locally. This may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In certain embodiments, it may be desirable to introduce the compound or a pharmaceutically acceptable salt thereof into the central nervous system by any suitable route, including intraventricular, intrathecal and epidural injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant. In certain embodiments, the compound and pharmaceutically acceptable salts thereof can be formulated as a suppository, with traditional binders and vehicles such as triglycerides.

In another embodiment, the compound and pharmaceutically acceptable salts thereof can be delivered in a vesicle, in particular a liposome (see Langer, 1990, Science 249:1527-1533; Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*).

In yet another embodiment, the compound and pharmaceutically acceptable salts thereof can be delivered in a controlled release system (see, *e.g.*, Goodson, in Medical

Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, Science 249:1527-1533) may be used. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507 Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled-release system can be placed in proximity of a target RNA of the compound or a pharmaceutically acceptable salt thereof, thus requiring only a fraction of the systemic dose.

Compositions comprising the compound or a pharmaceutically acceptable salt thereof ("compound compositions") can additionally comprise a suitable amount of a pharmaceutically acceptable vehicle so as to provide the form for proper administration to the patient.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, mammals, and more particularly in humans. The term "vehicle" refers to a diluent, adjuvant, excipient, or carrier with which a compound of the invention is administered. Such pharmaceutical vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical vehicles can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents may be used. When administered to a patient, the pharmaceutically acceptable vehicles are preferably sterile. Water is a preferred vehicle when the compound of the invention is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Compound compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Compound compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable vehicle is a capsule (see *e.g.*, U.S. Patent No. 5,698,155). Other examples of suitable pharmaceutical vehicles are described in Remington's Pharmaceutical Sciences, Alfonso R. Gennaro, ed., Mack Publishing Co. Easton, PA, 19th ed., 1995, pp. 1447 to 1676, incorporated herein by reference.

In a preferred embodiment, the compound or a pharmaceutically acceptable salt thereof is formulated in accordance with routine procedures as a pharmaceutical composition adapted for oral administration to human beings. Compositions for oral delivery may be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions may contain one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compositions. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time delay material such as glycerol monostearate or glycerol stearate may also be used. Oral compositions can include standard vehicles such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. Such vehicles are preferably of pharmaceutical grade. Typically, compositions for intravenous administration comprise sterile isotonic aqueous buffer. Where necessary, the compositions may also include a solubilizing agent.

In another embodiment, the compound or a pharmaceutically acceptable salt thereof can be formulated for intravenous administration. Compositions for intravenous administration may optionally include a local anesthetic such as lignocaine to lessen pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free

concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the compound or a pharmaceutically acceptable salt thereof is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the compound or a pharmaceutically acceptable salt thereof is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The amount of a compound or a pharmaceutically acceptable salt thereof that will be effective in the treatment of a particular disease will depend on the nature of the disease, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for oral administration are generally about 0.001 milligram to about 200 milligrams of a compound or a pharmaceutically acceptable salt thereof per kilogram body weight per day. In specific preferred embodiments of the invention, the oral dose is about 0.01 milligram to about 100 milligrams per kilogram body weight per day, more preferably about 0.1 milligram to about 75 milligrams per kilogram body weight per day, more preferably about 0.5 milligram to 5 milligrams per kilogram body weight per day. The dosage amounts described herein refer to total amounts administered; that is, if more than one compound is administered, or if a compound is administered with a therapeutic agent, then the preferred dosages correspond to the total amount administered. Oral compositions preferably contain about 10% to about 95% active ingredient by weight.

Suitable dosage ranges for intravenous (i.v.) administration are about 0.01 milligram to about 100 milligrams per kilogram body weight per day, about 0.1 milligram to about 35 milligrams per kilogram body weight per day, and about 1 milligram to about 10 milligrams per kilogram body weight per day. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight per day to about 1 mg/kg body weight per day. Suppositories generally contain about 0.01 milligram to about 50 milligrams of a compound of the invention per kilogram body weight per day and comprise active ingredient in the range of about 0.5% to about 10% by weight.

Recommended dosages for intradermal, intramuscular, intraperitoneal, subcutaneous, epidural, sublingual, intracerebral, intravaginal, transdermal administration or administration by inhalation are in the range of about 0.001 milligram to about 200

milligrams per kilogram of body weight per day. Suitable doses for topical administration are in the range of about 0.001 milligram to about 1 milligram, depending on the area of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Such animal models and systems are well known in the art.

The compound and pharmaceutically acceptable salts thereof are preferably assayed *in vitro* and *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays can be used to determine whether it is preferable to administer the compound, a pharmaceutically acceptable salt thereof, and/or another therapeutic agent. Animal model systems can be used to demonstrate safety and efficacy.

A variety of compounds can be used for treating or preventing diseases in mammals. Types of compounds include, but are not limited to, peptides, peptide analogs including peptides comprising non-natural amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphonic acids and α-amino phosphinic acids, or amino acids having non-peptide linkages, nucleic acids, nucleic acid analogs such as phosphorothioates or peptide nucleic acids ("PNAs"), hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose.

5. EXAMPLE: THERAPEUTIC TARGETS

The therapeutic targets presented herein are by way of example, and the present invention is not to be limited by the targets described herein. The therapeutic targets presented herein as DNA sequences are understood by one of skill in the art that the sequences can be converted to RNA sequences.

5.1. Tumor Necrosis Factor Alpha ("TNF-α")

GenBank Accession # X01394:

1 gcagaggacc agctaagagg gagagaagca actacagacc ccccctgaaa acaaccctca
61 gacgccacat cccctgacaa gctgccaggc aggttctctt cctctcacat actgacccac
121 ggctccaccc tctctcccct ggaaaggaca ccatgagcac tgaaagcatg atccgggacg
181 tggagctggc cgaggaggcg ctccccaaga agacaggggg gccccagggc tccaggcggt
241 gcttgttcct cagcctcttc tccttcctga tcgtggcagg cgccaccacg ctcttctgcc
301 tgctgcactt tggagtgatc ggcccccaga gggaagagtt ccccagggac ctctctctaa
361 tcagccctct ggcccaggca gtcagatcat cttctcgaac cccgagtgac aagcctgtag

421 cccatgttgt agcaaaccct caagctgagg ggcagctcca gtggctgaac cgccgggcca
481 atgccctcct ggccaatggc gtggagctga gagataacca gctggtggtg ccatcagagg
541 gcctgtacct catctactcc caggtcctct tcaagggcca aggctgcccc tccacccatg
601 tgctcctcac ccacaccatc agccgcatcg ccgtctccta ccagaccaag gtcaacctcc
661 tctctgccat caagagcccc tgccagaggg agaccccaga gggggctgag gccaagccct
721 ggtatgagcc catctatctg ggaggggtct tccagctgga gaagggtgac cgactcagcg
781 ctgagatcaa tcggcccgac tatctcgact ttgccgagtc tgggcaggtc tactttggga
841 tcattgccct gtgaggagga cgaacatcca accttcccaa acgcctcccc tgccccaatc
901 cctttattac cccctccttc agacaccctc aacctcttct ggctcaaaaa gagaattggg
961 ggcttagggt cggaacccaa gcttagaact ttaagcaaca agaccaccac ttcgaaacct
1021 gggattcagg aatgtgtggc ctgcacagtg aattgctggc aaccactaag aattcaaact
1081 ggggcctcca gaactcactg gggcctacag ctttgatccc tgacatctgg aatctggaga
1141 ccagggagcc tttggttctg gccagaatgc tgcaggactt gagaagacct cacctagaaa
1201 ttgacacaag tggaccttag gccttcctct ctccagatgt ttccagactt ccttgagaca
1261 cggagcccag ccctccccat ggagccagct ccctctattt atgtttgcac ttgtgattat
1321 ttattattta tttattattt atttatttac agatgaatgt atttatttgg gagaccgggg
1381 tatcctgggg gacccaatgt aggagctgcc ttggctcaga catgttttcc gtgaaaacgg
1441 agctgaacaa taggctgttc ccatgtagcc ccctggcctc tgtgccttct tttgattatg
1501 tttttaaaa tatttatctg attaagttgt ctaaacaatg ctgatttggt gaccaactgt
1561 cactcattgc tgagcctctg ctccccaggg gagttgtgtc tgtaatcgcc ctactattca
1621 gtggcgagaa ataaagtttg ctt (SEQ ID NO: 6)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 152
(2) 3' Untranslated Region - nts 852 - 1643

Initial Specific Target Motif:

Group I AU-Rich Element (ARE) Cluster in 3' untranslated region
5' AUUUAUUUAUUUAUUUAUUUA 3' (SEQ ID NO: 1)

5.2. <u>Granulocyte-macrophage Colony Stimulating Factor ("GM-CSF")</u>

GenBank Accession # NM_000758:

1 gctggaggat gtggctgcag agcctgctgc tcttgggcac tgtggcctgc agcatctctg
61 cacccgcccg ctcgcccagc cccagcacgc agccctggga gcatgtgaat gccatccagg
121 aggcccggcg tctcctgaac ctgagtagag acactgctgc tgagatgaat gaaacagtag

181 aagtcatctc agaaatgttt gacctccagg agccgacctg cctacagacc cgcctggagc
241 tgtacaagca gggcctgcgg ggcagcctca ccaagctcaa gggcccсttg accatgatgg
301 ccagccacta caagcagcac tgccctccaa ccccggaaac ttcctgtgca acccagacta
361 tcacctttga aagtttcaaa gagaacctga aggactttct gcttgtcatc ccctttgact
421 gctgggagcc agtccaggag tgagaccggc cagatgaggc tggccaagcc ggggagctgc
481 tctctcatga aacaagagct agaaactcag gatggtcatc ttggagggac caaggggtgg
541 gccacagcca tggtgggagt ggcctggacc tgccctgggc cacactgacc ctgatacagg
601 catggcagaa gaatgggaat attttatact gacagaaatc agtaatattt atatatttat
661 atttttaaaa tatttattta tttatttatt taagttcata ttccatattt attcaagatg
721 ttttaccgta ataattatta ttaaaaatat gcttct (SEQ ID NO: 7)

GenBank Accession # XM_003751:

1 tctggaggat gtggctgcag agcctgctgc tcttgggcac tgtggcctgc agcatctctg
61 cacccgcccg ctcgcccagc cccagcacgc agccctggga gcatgtgaat gccatccagg
121 aggcccggcg tctcctgaac ctgagtagag acactgctgc tgagatgaat gaaacagtag
181 aagtcatctc agaaatgttt gacctccagg agccgacctg cctacagacc cgcctggagc
241 tgtacaagca gggcctgcgg ggcagcctca ccaagctcaa gggcccсttg accatgatgg
301 ccagccacta caagcagcac tgccctccaa ccccggaaac ttcctgtgca acccagacta
361 tcacctttga aagtttcaaa gagaacctga aggactttct gcttgtcatc ccctttgact
421 gctgggagcc agtccaggag tgagaccggc cagatgaggc tggccaagcc ggggagctgc
481 tctctcatga aacaagagct agaaactcag gatggtcatc ttggagggac caaggggtgg
541 gccacagcca tggtgggagt ggcctggacc tgccctgggc cacactgacc ctgatacagg
601 catggcagaa gaatgggaat attttatact gacagaaatc agtaatattt atatatttat
661 atttttaaaa tatttattta tttatttatt taagttcata ttccatattt attcaagatg
721 ttttaccgta ataattatta ttaaaaatat gcttct (SEQ ID NO: 8)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 32
(2) 3' Untranslated Region - nts 468 - 789

Initial Specific Target Motif:

Group I AU-Rich Element (ARE) Cluster in 3' untranslated region
5' AUUUAUUUAUUUAUUUAUUUA 3' (SEQ ID NO: 1)

5.3. Interleukin 2 ("IL-2")

GenBank Accession # U25676:

1 atcactctct ttaatcacta ctcacattaa cctcaactcc tgccacaatg tacaggatgc
61 aactcctgtc ttgcattgca ctaattcttg cacttgtcac aaacagtgca cctacttcaa
121 gttcgacaaa gaaaacaaag aaaacacagc tacaactgga gcatttactg ctggatttac
181 agatgatttt gaatggaatt aataattaca agaatcccaa actcaccagg atgctcacat
241 ttaagtttta catgcccaag aaggccacag aactgaaaca gcttcagtgt ctagaagaag
301 aactcaaacc tctggaggaa gtgctgaatt tagctcaaag caaaaacttt cacttaagac
361 ccagggactt aatcagcaat atcaacgtaa tagttctgga actaaaggga tctgaaacaa
421 cattcatgtg tgaatatgca gatgagacag caaccattgt agaatttctg aacagatgga
481 ttacctttttg tcaaagcatc atctcaacac taacttgata attaagtgct tcccacttaa
541 aacatatcag gccttctatt tatttattta aatatttaaa ttttatattt attgttgaat
601 gtatggttgc tacctattgt aactattatt cttaatctta aaactataaa tatggatctt
661 ttatgattct ttttgtaagc cctaggggct ctaaaatggt ttaccttatt tatcccaaaa
721 atatttatta ttatgttgaa tgttaaatat agtatctatg tagattggtt agtaaaacta
781 tttaataaat ttgataaata taaaaaaaaa aaacaaaaaa aaaaa (SEQ ID NO: 9)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 47
(2) 3' Untranslated Region - nts 519- 825

Initial Specific Target Motifs:

Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

5.4. Interleukin 6 ("IL-6")

GenBank Accession # NM_000600:

1 ttctgccctc gagcccaccg ggaacgaaag agaagctcta tctcgcctcc aggagcccag
61 ctatgaactc cttctccaca agcgccttcg gtccagttgc cttctccctg gggctgctcc
121 tggtgttgcc tgctgccttc cctgccccag tacccccagg agaagattcc aaagatgtag
181 ccgccccaca cagacagcca ctcacctctt cagaacgaat tgacaaacaa attcggtaca
241 tcctcgacgg catctcagcc ctgagaaagg agacatgtaa caagagtaac atgtgtgaaa
301 gcagcaaaga ggcactggca gaaaacaacc tgaaccttcc aaagatggct gaaaaagatg
361 gatgcttcca atctggattc aatgaggaga cttgcctggt gaaaatcatc actggtcttt
421 tggagtttga ggtataccta gagtacctcc agaacagatt tgagagtagt gaggaacaag

481 ccagagctgt gcagatgagt acaaaagtcc tgatccagtt cctgcagaaa aaggcaaaga
541 atctagatgc aataaccacc cctgacccaa ccacaaatgc cagcctgctg acgaagctgc
601 aggcacagaa ccagtggctg caggacatga caactcatct cattctgcgc agctttaagg
661 agttcctgca gtccagcctg agggctcttc ggcaaatgta gcatgggcac ctcagattgt
721 tgttgttaat gggcattcct tcttctggtc agaaacctgt ccactgggca cagaacttat
781 gttgttctct atggagaact aaaagtatga gcgttaggac actattttaa ttatttttaa
841 tttattaata tttaaatatg tgaagctgag ttaatttatg taagtcatat ttatattttt
901 aagaagtacc acttgaaaca ttttatgtat tagttttgaa ataataatgg aaagtggcta
961 tgcagtttga atatcctttg tttcagagcc agatcatttc ttggaaagtg taggcttacc
1021 tcaaataaat ggctaactta tacatatttt taaagaaata tttatattgt atttatataa
1081 tgtataaatg gtttttatac caataaatgg cattttaaaa aattc (SEQ ID NO: 11)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 62
(2) 3' Untranslated Region - nts 699 - 1125

Initial Specific Target Motifs:

Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

5.5. <u>Vascular Endothelial Growth Factor ("VEGF")</u>

GenBank Accession # AF022375:

1 aagagctcca gagagaagtc gaggaagaga gagacggggt cagagagagc gcgcgggcgt
61 gcgagcagcg aaagcgacag gggcaaagtg agtgacctgc ttttgggggt gaccgccgga
121 gcgcggcgtg agccctcccc cttgggatcc cgcagctgac cagtcgcgct gacggacaga
181 cagacagaca ccgcccccag ccccagttac cacctcctcc ccggccggcg gcggacagtg
241 gacgcggcgg cgagccgcgg gcaggggccg gagcccgccc ccggaggcgg ggtggagggg
301 gtcggagctc gcggcgtcgc actgaaactt ttcgtccaac ttctgggctg ttctcgcttc
361 ggaggagccg tggtccgcgc gggggaagcc gagccgagcg gagccgcgag aagtgctagc
421 tcgggccggg aggagccgca gccggaggag ggggaggagg aagaagagaa ggaagaggag
481 agggggccgc agtggcgact cggcgctcgg aagccgggct catggacggg tgaggcggcg
541 gtgtgcgcag acagtgctcc agcgcgcgcg ctccccagcc ctggcccggc ctcgggccgg
601 gaggaagagt agctcgccga ggcgccgagg agagcgggcc gccccacagc ccgagccgga
661 gagggacgcg agccgcgcgc cccggtcggg cctccgaaac catgaacttt ctgctgtctt
721 gggtgcattg gagccttgcc ttgctgctct acctccacca tgccaagtgg tcccaggctg

781 cacccatggc agaaggagga gggcagaatc atcacgaagt ggtgaagttc atggatgtct
841 atcagcgcag ctactgccat ccaatcgaga ccctggtgga catcttccag gagtaccctg
901 atgagatcga gtacatcttc aagccatcct gtgtgcccct gatgcgatgc gggggctgct
961 ccaatgacga gggcctggag tgtgtgccca ctgaggagtc caacatcacc atgcagatta
1021 tgcggatcaa acctcaccaa ggccagcaca taggagagat gagcttccta cagcacaaca
1081 aatgtgaatg cagaccaaag aaagatagag caagacaaga aaatccctgt gggccttgct
1141 cagagcggag aaagcatttg tttgtacaag atccgcagac gtgtaaatgt tcctgcaaaa
1201 acacacactc gcgttgcaag gcgaggcagc ttgagttaaa cgaacgtact tgcagatgtg
1261 acaagccgag gcggtgagcc gggcaggagg aaggagcctc cctcagggtt tcgggaacca
1321 gatctctctc caggaaagac tgatacagaa cgatcgatac agaaaccacg ctgccgccac
1381 cacaccatca ccatcgacag aacagtcctt aatccagaaa cctgaaatga aggaagagga
1441 gactctgcgc agagcacttt gggtccggag ggcgagactc cggcggaagc attcccgggc
1501 gggtgaccca gcacggtccc tcttggaatt ggattcgcca ttttattttt cttgctgcta
1561 aatcaccgag cccggaagat tagagagttt tatttctggg attcctgtag acacacccac
1621 ccacatacat acatttatat atatatatat tatatatata taaaaataaa tatctctatt
1681 ttatatatat aaaatatata tattcttttt ttaaattaac agtgctaatg ttattggtgt
1741 cttcactgga tgtatttgac tgctgtggac ttgagttggg aggggaatgt tcccactcag
1801 atcctgacag ggaagaggag gagatgagag actctggcat gatctttttt ttgtcccact
1861 tggtggggcc agggtcctct cccctgccca agaatgtgca aggccagggc atgggggcaa
1921 atatgaccca gttttgggaa caccgacaaa cccagccctg gcgctgagcc tctctacccc
1981 aggtcagacg gacagaaaga caaatcacag gttccgggat gaggacaccg gctctgacca
2041 ggagtttggg gagcttcagg acattgctgt gctttgggga ttccctccac atgctgcacg
2101 cgcatctcgc ccccaggggc actgcctgga agattcagga gcctgggcgg ccttcgctta
2161 ctctcacctg cttctgagtt gcccaggagg ccactggcag atgtcccggc gaagagaaga
2221 gacacattgt tggaagaagc agcccatgac agcgcccctt cctgggactc gccctcatcc
2281 tcttcctgct ccccttcctg gggtgcagcc taaaaggacc tatgtcctca caccattgaa
2341 accactagtt ctgtcccccc aggaaacctg gttgtgtgtg tgtgagtggt tgaccttcct
2401 ccatcccctg gtccttccct tcccttcccg aggcacagag agacagggca ggatccacgt
2461 gcccattgtg gaggcagaga aaagagaaag tgttttatat acggtactta tttaatatcc
2521 ctttttaatt agaaattaga acagttaatt taattaaaga gtagggtttt ttttcagtat
2581 tcttggttaa tatttaattt caactattta tgagatgtat cttttgctct ctcttgctct
2641 cttatttgta ccggtttttg tatataaaat tcatgtttcc aatctctctc tccctgatcg
2701 gtgacagtca ctagcttatc ttgaacagat atttaatttt gctaacactc agctctgccc
2761 tccccgatcc cctggctccc cagcacacat tcctttgaaa gagggtttca atatacatct
2821 acatactata tatatattgg gcaacttgta tttgtgtgta tatatatata tatatgttta

2881 tgtatatatg tgatcctgaa aaaataaaca tcgctattct gtttttata tgttcaaacc
2941 aaacaagaaa aaatagagaa ttctacatac taaatctctc tcctttttta attttaatat
3001 ttgttatcat ttatttattg gtgctactgt ttatccgtaa taattgtggg gaaaagatat
3061 taacatcacg tctttgtctc tagtgcagtt tttcgagata ttccgtagta catatttatt
3121 tttaaacaac gacaaagaaa tacagatata tcttaaaaaa aaaaaa (SEQ ID NO: 12)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 701
(2) 3' Untranslated Region - nts 1275 - 3166

Initial Specific Target Motifs:

(1) Internal Ribosome Entry Site (IRES) in 5' untranslated region nts 513 -704
5'CCGGGCUCAUGGACGGGUGAGGCGGCGGUGUGCGCAGACAGUG
CUCCAGCGCGCGCGCUCCCCAGCCCUGGCCCGGCCUCGGGCCGGG
AGGAAGAGUAGCUCGCCGAGGCGCCGAGGAGAGCGGGCCGCCCC
ACAGCCCGAGCCGGAGAGGGACGCGAGCCGCGCGCCCCGGUCGG
GCCUCCGAAACCAUGAACUUUCUGCUGUCUUGGGUGCAUUGGAG
CCUUGCCUUGCUGCUCUACCUCCACCAUG 3' (SEQ ID NO: 13)
(2) Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

5.6. <u>Human Immunodeficiency Virus I ("HIV-1")</u>

GenBank Accession # NC_001802:

1 ggtctctctg gttagaccag atctgagcct gggagctctc tggctaacta gggaacccac
61 tgcttaagcc tcaataaagc ttgccttgag tgcttcaagt agtgtgtgcc cgtctgttgt
121 gtgactctgg taactagaga tccctcagac ccttttagtc agtgtggaaa atctctagca
181 gtggcgcccg aacagggacc tgaaagcgaa agggaaacca gaggagctct ctcgacgcag
241 gactcggctt gctgaagcgc gcacggcaag aggcgagggg cggcgactgg tgagtacgcc
301 aaaaattttg actagcggag gctagaagga gagagatggg tgcgagagcg tcagtattaa
361 gcgggggaga attagatcga tgggaaaaaa ttcggttaag gccaggggga aagaaaaaat
421 ataaattaaa acatatagta tgggcaagca gggagctaga acgattcgca gttaatcctg
481 gcctgttaga aacatcagaa ggctgtagac aaatactggg acagctacaa ccatcccttc
541 agacaggatc agaagaactt agatcattat ataatacagt agcaaccctc tattgtgtgc
601 atcaaaggat agagataaaa gacaccaagg aagctttaga caagatagag gaagagcaaa
661 acaaaagtaa gaaaaaagca cagcaagcag cagctgacac aggacacagc aatcaggtca

721 gccaaaatta ccctatagtg cagaacatcc aggggcaaat ggtacatcag gccatatcac
781 ctagaacttt aaatgcatgg gtaaaagtag tagaagagaa ggctttcagc ccagaagtga
841 tacccatgtt ttcagcatta tcagaaggag ccaccccaca agatttaaac accatgctaa
901 acacagtggg gggacatcaa gcagccatgc aaatgttaaa agagaccatc aatgaggaag
961 ctgcagaatg ggatagagtg catccagtgc atgcagggcc tattgcacca ggccagatga
1021 gagaaccaag gggaagtgac atagcaggaa ctactagtac ccttcaggaa caaataggat
1081 ggatgacaaa taatccacct atcccagtag gagaaattta taaaagatgg ataatcctgg
1141 gattaaataa aatagtaaga atgtatagcc ctaccagcat tctggacata agacaaggac
1201 caaaggaacc ctttagagac tatgtagacc ggttctataa aactctaaga gccgagcaag
1261 cttcacagga ggtaaaaaat tggatgacag aaaccttgtt ggtccaaaat gcgaacccag
1321 attgtaagac tattttaaaa gcattgggac cagcggctac actagaagaa atgatgacag
1381 catgtcaggg agtaggagga cccggccata aggcaagagt tttggctgaa gcaatgagcc
1441 aagtaacaaa ttcagctacc ataatgatgc agagaggcaa ttttaggaac caaagaaaga
1501 ttgttaagtg tttcaattgt ggcaaagaag ggcacacagc cagaaattgc agggccccta
1561 ggaaaaaggg ctgttggaaa tgtggaaagg aaggacacca aatgaaagat tgtactgaga
1621 gacaggctaa ttttttaggg aagatctggc cttcctacaa gggaaggcca gggaattttc
1681 ttcagagcag accagagcca acagccccac cagaagagag cttcaggtct ggggtagaga
1741 caacaactcc ccctcagaag caggagccga tagacaagga actgtatcct ttaacttccc
1801 tcaggtcact ctttggcaac gacccctcgt cacaataaag atagggggc aactaaagga
1861 agctctatta gatacaggag cagatgatac agtattagaa gaaatgagtt tgccaggaag
1921 atggaaacca aaaatgatag ggggaattgg aggttttatc aaagtaagac agtatgatca
1981 gatactcata gaaatctgtg gacataaagc tataggtaca gtattagtag gacctacacc
2041 tgtcaacata attggaagaa atctgttgac tcagattggt tgcactttaa attttcccat
2101 tagccctatt gagactgtac cagtaaaatt aaagccagga atggatggcc caaaagttaa
2161 acaatggcca ttgacagaag aaaaaataaa agcattagta gaaatttgta cagagatgga
2221 aaaggaaggg aaaatttcaa aaattgggcc tgaaaatcca tacaatactc cagtatttgc
2281 cataaagaaa aaagacagta ctaaatggag aaaattagta gatttcagag aacttaataa
2341 gagaactcaa gacttctggg aagttcaatt aggaatacca catcccgcag ggttaaaaaa
2401 gaaaaaatca gtaacagtac tggatgtggg tgatgcatat ttttcagttc ccttagatga
2461 agacttcagg aagtatactg catttaccat acctagtata aacaatgaga caccagggat
2521 tagatatcag tacaatgtgc ttccacaggg atggaaagga tcaccagcaa tattccaaag
2581 tagcatgaca aaaatcttag agccttttag aaaacaaaat ccagacatag ttatctatca
2641 atacatggat gatttgtatg taggatctga cttagaaata gggcagcata gaacaaaaat
2701 agaggagctg agacaacatc tgttgaggtg gggacttacc acaccagaca aaaaacatca
2761 gaaagaacct ccattccttt ggatgggtta tgaactccat cctgataaat ggacagtaca

2821 gcctatagtg ctgccagaaa aagacagctg gactgtcaat gacatacaga agttagtggg
2881 gaaattgaat tgggcaagtc agatttaccc agggattaaa gtaaggcaat tatgtaaact
2941 ccttagagga accaaagcac taacagaagt aataccacta acagaagaag cagagctaga
3001 actggcagaa aacagagaga ttctaaaaga accagtacat ggagtgtatt atgacccatc
3061 aaaagactta atagcagaaa tacagaagca ggggcaaggc caatggacat atcaaattta
3121 tcaagagcca tttaaaaatc tgaaaacagg aaaatatgca agaatgaggg gtgcccacac
3181 taatgatgta aaacaattaa cagaggcagt gcaaaaaata accacagaaa gcatagtaat
3241 atggggaaag actcctaaat ttaaactgcc catacaaaag gaaacatggg aaacatggtg
3301 gacagagtat tggcaagcca cctggattcc tgagtgggag tttgttaata cccctcccct
3361 agtgaaatta tggtaccagt tagagaaaga acccatagta ggagcagaaa ccttctatgt
3421 agatggggca gctaacaggg agactaaatt aggaaaagca ggatatgtta ctaatagagg
3481 aagacaaaaa gttgtcaccc taactgacac aacaaatcag aagactgagt tacaagcaat
3541 ttatctagct ttgcaggatt cgggattaga agtaaacata gtaacagact cacaatatgc
3601 attaggaatc attcaagcac aaccagatca aagtgaatca gagttagtca atcaaataat
3661 agagcagtta ataaaaaagg aaaaggtcta tctggcatgg gtaccagcac acaaaggaat
3721 tggaggaaat gaacaagtag ataaattagt cagtgctgga atcaggaaag tactattttt
3781 agatggaata gataaggccc aagatgaaca tgagaaatat cacagtaatt ggagagcaat
3841 ggctagtgat tttaacctgc cacctgtagt agcaaaagaa atagtagcca gctgtgataa
3901 atgtcagcta aaaggagaag ccatgcatgg acaagtagac tgtagtccag gaatatggca
3961 actagattgt acacatttag aaggaaaagt tatcctggta gcagttcatg tagccagtgg
4021 atatatagaa gcagaagtta ttccagcaga aacagggcag gaaacagcat attttctttt
4081 aaaattagca ggaagatggc cagtaaaaac aatacatact gacaatggca gcaatttcac
4141 cggtgctacg gttagggccg cctgttggtg ggcgggaatc aagcaggaat ttggaattcc
4201 ctacaatccc caaagtcaag gagtagtaga atctatgaat aaagaattaa agaaaattat
4261 aggacaggta agagatcagg ctgaacatct taagacagca gtacaaatgg cagtattcat
4321 ccacaatttt aaaagaaaag gggggattgg ggggtacagt gcaggggaaa gaatagtaga
4381 cataatagca acagacatac aaactaaaga attacaaaaa caaattacaa aaattcaaaa
4441 ttttcgggtt tattacaggg acagcagaaa tccactttgg aaaggaccag caaagctcct
4501 ctggaaaggt gaaggggcag tagtaataca agataatagt gacataaaag tagtgccaag
4561 aagaaaagca aagatcatta gggattatgg aaaacagatg gcaggtgatg attgtgtggc
4621 aagtagacag gatgaggatt agaacatgga aaagtttagt aaaacaccat atgtatgttt
4681 cagggaaagc taggggatgg ttttatagac atcactatga aagccctcat ccaagaataa
4741 gttcagaagt acacatccca ctaggggatg ctagattggt aataacaaca tattggggtc
4801 tgcatacagg agaaagagac tggcatttgg gtcagggagt ctccatagaa tggaggaaaa
4861 agagatatag cacacaagta gaccctgaac tagcagacca actaattcat ctgtattact

4921 ttgactgttt ttcagactct gctataagaa aggccttatt aggacacata gttagcccta
4981 ggtgtgaata tcaagcagga cataacaagg taggatctct acaatacttg gcactagcag
5041 cattaataac accaaaaaag ataaagccac ctttgcctag tgttacgaaa ctgacagagg
5101 atagatggaa caagccccag aagaccaagg gccacagagg gagccacaca atgaatggac
5161 actagagctt ttagaggagc ttaagaatga agctgttaga cattttccta ggatttggct
5221 ccatggctta gggcaacata tctatgaaac ttatggggat acttgggcag gagtggaagc
5281 cataataaga attctgcaac aactgctgtt tatccatttt cagaattggg tgtcgacata
5341 gcagaatagg cgttactcga cagaggagag caagaaatgg agccagtaga tcctagacta
5401 gagccctgga agcatccagg aagtcagcct aaaactgctt gtaccaattg ctattgtaaa
5461 aagtgttgct ttcattgcca agtttgtttc ataacaaaag ccttaggcat ctcctatggc
5521 aggaagaagc ggagacagcg acgaagagct catcagaaca gtcagactca tcaagcttct
5581 ctatcaaagc agtaagtagt acatgtaatg caacctatac caatagtagc aatagtagca
5641 ttagtagtag caataataat agcaatagtt gtgtggtcca tagtaatcat agaatatagg
5701 aaaatattaa gacaaagaaa aatagacagg ttaattgata gactaataga aagagcagaa
5761 gacagtggca atgagagtga aggagaaata tcagcacttg tggagatggg ggtggagatg
5821 gggcaccatg ctccttggga tgttgatgat ctgtagtgct acagaaaaat tgtgggtcac
5881 agtctattat ggggtacctg tgtggaagga agcaaccacc actctatttt gtgcatcaga
5941 tgctaaagca tatgatacag aggtacataa tgtttgggcc acacatgcct gtgtacccac
6001 agaccccaac ccacaagaag tagtattggt aaatgtgaca gaaaatttta acatgtggaa
6061 aaatgacatg gtagaacaga tgcatgagga tataatcagt ttatgggatc aaagcctaaa
6121 gccatgtgta aaattaaccc cactctgtgt tagtttaaag tgcactgatt tgaagaatga
6181 tactaatacc aatagtagta gcgggagaat gataatggag aaaggagaga taaaaaactg
6241 ctctttcaat atcagcacaa gcataagagg taaggtgcag aaagaatatg catttttta
6301 taaacttgat ataataccaa tagataatga tactaccagc tataagttga caagttgtaa
6361 cacctcagtc attacacagg cctgtccaaa ggtatccttt gagccaattc ccatacatta
6421 ttgtgccccg gctggttttg cgattctaaa atgtaataat aagacgttca atggaacagg
6481 accatgtaca aatgtcagca cagtacaatg tacacatgga attaggccag tagtatcaac
6541 tcaactgctg ttaaatggca gtctagcaga agaagaggta gtaattagat ctgtcaattt
6601 cacggacaat gctaaaacca taatagtaca gctgaacaca tctgtagaaa ttaattgtac
6661 aagacccaac aacaatacaa gaaaaagaat ccgtatccag agaggaccag ggagagcatt
6721 tgttacaata ggaaaaatag gaaatatgag acaagcacat tgtaacatta gtagagcaaa
6781 atggaataac actttaaaac agatagctag caaattaaga gaacaatttg gaaataataa
6841 aacaataatc tttaagcaat cctcaggagg ggacccagaa attgtaacgc acagttttaa
6901 ttgtggaggg gaatttttct actgtaattc aacacaactg tttaatagta cttggtttaa
6961 tagtacttgg agtactgaag ggtcaaataa cactgaagga agtgacacaa tcaccctccc

7021 atgcagaata aaacaaatta taaacatgtg gcagaaagta ggaaaagcaa tgtatgcccc
7081 tcccatcagt ggacaaatta gatgttcatc aaatattaca gggctgctat taacaagaga
7141 tggtggtaat agcaacaatg agtccgagat cttcagacct ggaggaggag atatgaggga
7201 caattggaga agtgaattat ataaatataa agtagtaaaa attgaaccat taggagtagc
7261 acccaccaag gcaaagagaa gagtggtgca gagagaaaaa agagcagtgg gaataggagc
7321 tttgttcctt gggttcttgg gagcagcagg aagcactatg ggcgcagcct caatgacgct
7381 gacggtacag gccagacaat tattgtctgg tatagtgcag cagcagaaca atttgctgag
7441 ggctattgag gcgcaacagc atctgttgca actcacagtc tggggcatca agcagctcca
7501 ggcaagaatc ctggctgtgg aaagatacct aaaggatcaa cagctcctgg ggatttgggg
7561 ttgctctgga aaactcattt gcaccactgc tgtgccttgg aatgctagtt ggagtaataa
7621 atctctggaa cagatttgga atcacacgac ctggatggag tgggacagag aaattaacaa
7681 ttacacaagc ttaatacact ccttaattga agaatcgcaa aaccagcaag aaaagaatga
7741 acaagaatta ttggaattag ataaatgggc aagtttgtgg aattggttta acataacaaa
7801 ttggctgtgg tatataaaat tattcataat gatagtagga ggcttggtag gtttaagaat
7861 agtttttgct gtactttcta tagtgaatag agttaggcag ggatattcac cattatcgtt
7921 tcagacccac ctcccaaccc cgaggggacc cgacaggccc gaaggaatag aagaagaagg
7981 tggagagaga gacagagaca gatccattcg attagtgaac ggatccttgg cacttatctg
8041 ggacgatctg cggagcctgt gcctcttcag ctaccaccgc ttgagagact tactcttgat
8101 tgtaacgagg attgtggaac ttctgggacg cagggggtgg gaagccctca aatattggtg
8161 gaatctccta cagtattgga gtcaggaact aaagaatagt gctgttagct tgctcaatgc
8221 cacagccata gcagtagctg aggggacaga tagggttata gaagtagtac aaggagcttg
8281 tagagctatt cgccacatac ctagaagaat aagacagggc ttggaaagga ttttgctata
8341 agatgggtgg caagtggtca aaaagtagtg tgattggatg gcctactgta agggaaagaa
8401 tgagacgagc tgagccagca gcagataggg tgggagcagc atctcgagac ctggaaaaac
8461 atggagcaat cacaagtagc aatacagcag ctaccaatgc tgcttgtgcc tggctagaag
8521 cacaagagga ggaggaggtg ggttttccag tcacacctca ggtaccttta agaccaatga
8581 cttacaaggc agctgtagat cttagccact ttttaaaaga aaagggggga ctggaagggc
8641 taattcactc ccaaagaaga caagatatcc ttgatctgtg gatctaccac acacaaggct
8701 acttccctga ttagcagaac tacacaccag ggccaggggt cagatatcca ctgacctttg
8761 gatggtgcta caagctagta ccagttgagc cagataagat agaagaggcc aataaaggag
8821 agaacaccag cttgttacac cctgtgagcc tgcatgggat ggatgacccg gagagagaag
8881 tgttagagtg gaggtttgac agccgcctag catttcatca cgtggcccga gagctgcatc
8941 cggagtactt caagaactgc tgacatcgag cttgctacaa gggactttcc gctggggact
9001 ttccagggag gcgtggcctg ggcgggactg gggagtggcg agccctcaga tcctgcatat
9061 aagcagctgc tttttgcctg tactgggtct ctctggttag accagatctg agcctgggag

9121 ctctctggct aactagggaa cccactgctt aagcctcaat aaagcttgcc ttgagtgctt
9181 c (SEQ ID NO: 14)

Initial Specific Target Motifs:

(1) Trans-activation response region/Tat protein binding site - TAR RNA - nts 1 - 60
"Minimal" TAR RNA element
5' GGCAGAUCUGAGCCUGGGAGCUCUCUGCC 3' (SEQ ID NO: 15)

(2) Gag/Pol Frameshifting Site - "Minimal" frameshifting element
5' UUUUUUAGGGAAGAUCUGGCCUUCCUACAAGGGAAGGCCAGG GAAUUUUCUU 3' (SEQ ID NO: 16)

5.7. Hepatitis C Virus ("HCV" - Genotypes 1a & 1b)

GenBank Accession # NC_001433:

1 ttgggggcga cactccacca tagatcactc ccctgtgagg aactactgtc ttcacgcaga
61 aagcgtctag ccatggcgtt agtatgagtg ttgtgcagcc tccaggaccc cccctcccgg
121 gagagccata gtggtctgcg gaaccggtga gtacaccgga attgccagga cgaccgggtc
181 ctttcttgga tcaacccgct caatgcctgg agatttgggc gtgcccccgc gagactgcta
241 gccgagtagt gttgggtcgc gaaaggcctt gtggtactgc ctgatagggt gcttgcgagt
301 gccccgggag gtctcgtaga ccgtgcatca tgagcacaaa tcctaaacct caaagaaaaa
361 ccaaacgtaa caccaaccgc cgcccacagg acgttaagtt cccgggcggt ggtcagatcg
421 ttggtggagt ttacctgttg ccgcgcaggg gccccaggtt gggtgtgcgc gcgactagga
481 agacttccga gcggtcgcaa cctcgtggaa ggcgacaacc tatccccaag gctcgccggc
541 ccgagggtag gacctgggct cagcccgggt acccttggcc cctctatggc aacgagggta
601 tggggtgggc aggatggctc ctgtcacccc gtggctctcg gcctagttgg ggccccacag
661 accccggcg taggtcgcgt aatttgggta aggtcatcga tacccttaca tgcggcttcg
721 ccgacctcat ggggtacatt ccgcttgtcg gcgcccccct agggggcgct gccagggccc
781 tggcacatgg tgtccgggtt ctggaggacg gcgtgaacta tgcaacaggg aatctgcccg
841 gttgctcttt ctctatcttc ctcttagctt tgctgtcttg tttgaccatc ccagcttccg
901 cttacgaggt gcgcaacgtg tccgggatat accatgtcac gaacgactgc tccaactcaa
961 gtattgtgta tgaggcagcg gacatgatca tgcacacccc cgggtgcgtg ccctgcgtcc
1021 gggagagtaa tttctcccgt tgctgggtag cgctcactcc cacgctcgcg gccaggaaca
1081 gcagcatccc caccacgaca atacgacgcc acgtcgattt gctcgttggg gcggctgctc
1141 tctgttccgc tatgtacgtt ggggatctct gcggatccgt ttttctcgtc tcccagctgt
1201 tcaccttctc acctcgccgg tatgagacgg tacaagattg caattgctca atctatcccg

1261 gccacgtatc aggtcaccgc atggcttggg atatgatgat gaactggtca cctacaacgg
1321 ccctagtggt atcgcagcta ctccggatcc cacaagccgt cgtggacatg gtggcggggg
1381 cccactgggg tgtcctagcg ggccttgcct actattccat ggtggggaac tgggctaagg
1441 tcttgattgt gatgctactc tttgctggcg ttgacgggca caccccacgtg acagggggaa
1501 gggtagcctc cagcacccag agcctcgtgt cctggctctc acaaggccca tctcagaaaa
1561 tccaactcgt gaacaccaac ggcagctggc acatcaacag gaccgctctg aattgcaatg
1621 actccctcca aactgggttc attgctgcgc tgttctacgc acacaggttc aacgcgtccg
1681 ggtgcccaga gcgcatggct agctgccgcc ccatcgatga gttcgctcag gggtggggtc
1741 ccatcactca tgatatgcct gagagctcgg accagaggcc atattgctgg cactacgcgc
1801 ctcgaccgtg cgggatcgtg cctgcgtcgc aggtgtgtgg tccagtgtat tgcttcactc
1861 cgagccctgt tgtagtgggg acgaccgatc gtttcggcgc tcctacgtat agctgggggg
1921 agaatgagac agacgtgctg ctacttagca acacgcggcc gcctcaaggc aactggtttg
1981 ggtgcacgtg gatgaacagc actgggttca ccaagacgtg cgggggccct ccgtgcaaca
2041 tcgggggggt cggcaacaac accttggtct gccccacgga ttgcttccgg aagcaccccg
2101 aggccactta cacaaagtgt ggctcggggc cctggttgac acccaggtgc atggttgact
2161 acccatacag gctctggcac taccccctgca ctgttaactt taccgtcttt aaggtcagga
2221 tgtatgtggg gggcgtggag cacaggctca atgctgcatg caattggact cgaggagagc
2281 gctgtgactt ggaggacagg gataggtcag aactcagccc gctgctgctg tctacaacag
2341 agtggcagat actgccctgt tccttcacca ccctaccggc cctgtccact ggcttgatcc
2401 atcttcaccg gaacatcgtg gacgtgcaat acctgtacgg tatagggtcg gcagttgtct
2461 cctttgcaat caaatgggag tatatcctgt tgcttttcct tcttctggcg gacgcgcgcg
2521 tctgtgcctg cttgtggatg atgctgctga tagcccaggc tgaggccacc ttagagaacc
2581 tggtggtcct caatgcggcg tctgtggccg gagcgcatgg ccttctctcc ttcctcgtgt
2641 tcttctgcgc cgcctggtac atcaaaggca ggctggtccc tggggcggca tatgctctct
2701 atggcgtatg gccgttgctc ctgctcttgc tggccttacc accacgagct tatgccatgg
2761 accgagagat ggctgcatcg tgcggaggcg cggtttttgt aggtctggta ctcttgacct
2821 tgtcaccata ctataaggtg ttcctcgcta ggctcatatg gtggttacaa tattttatca
2881 ccagagccga ggcgcacttg caagtgtggg tccccccctct caatgttcgg ggaggccgcg
2941 atgccatcat cctccttaca tgcgcggtcc atccagagct aatctttgac atcaccaaac
3001 tcctgctcgc catactcggt ccgctcatgg tgctccaggc tggcataact agagtgccgt
3061 actttgtacg cgctcagggg ctcatccgtg catgcatgtt agtgcggaag gtcgctggag
3121 gccactatgt ccaaatggcc ttcatgaagc tggccgcgct gacaggtacg tacgtatatg
3181 accatcttac tccactgcgg gattgggccc acgcgggcct acgagacctt gcggtggcag
3241 tagagcccgt cgtcttctct gacatggaga ctaaactcat cacctggggg gcagacaccg
3301 cggcgtgtgg ggacatcatc tcgggtctac cagtctccgc ccgaaggggg aaggagatac

3361 ttctaggacc ggccgatagt tttggagagc aggggtggcg gctccttgcg cctatcacgg
3421 cctattccca acaaacgcgg ggcctgcttg gctgtatcat cactagcctc acaggtcggg
3481 acaagaacca ggtcgatggg gaggttcagg tgctctccac cgcaacgcaa tctttcctgg
3541 cgacctgcgt caatggcgtg tgttggaccg tctaccatgg tgccggctcg aagaccctgg
3601 ccggcccgaa gggtccaatc acccaaatgt acaccaatgt agaccaggac ctcgtcggct
3661 ggccggcgcc ccccggggcg cgctccatga caccgtgcac ctgcggcagc tcggaccttt
3721 acttggtcac gaggcatgct gatgtcgttc cggtgcgccg gcggggcgac agcaggggga
3781 gcctgctttc ccccaggccc atctcctacc tgaagggctc ctcgggtgga ccactgcttt
3841 gcccttcggg gcacgttgta ggcatcttcc gggctgctgt gtgcacccgg ggggttgcga
3901 aggcggtgga cttcataccc gttgagtcta tggaaactac catgcggtct ccggtcttca
3961 cagacaactc atcccctccg gccgtaccgc aaacattcca agtggcacat ttacacgctc
4021 ccactggcag cggcaagagc accaaagtgc cggctgcata tgcagcccaa gggtacaagg
4081 tgctcgtcct aaacccgtcc gttgccgcca cattgggctt tggagcgtat atgtccaagg
4141 cacatggcat cgagcctaac atcagaactg ggtaaggac catcaccacg ggcggcccca
4201 tcacgtactc cacctattgc aagttccttg ccgacggtgg atgctccggg ggcgcctatg
4261 acatcataat atgtgatgaa tgccactcaa ctgactcgac taccatcttg ggcatcggca
4321 cagtcctgga tcaggcagag acggctggag cgcggctcgt cgtgctcgcc accgccacgc
4381 ctccgggatc gatcaccgtg ccacacccca acatcgagga agtggccctg tccaacactg
4441 gagagattcc cttctatggc aaagccatcc ccattgaggc catcaagggg ggaaggcatc
4501 tcatcttctg ccattccaag aagaagtgtg acgagctcgc cgcaaagctg acaggcctcg
4561 gactcaatgc tgtagcgtat taccggggtc tcgatgtgtc cgtcataccg actagcggag
4621 acgtcgttgt cgtggcaaca gacgctctaa tgacgggttt taccggcgac tttgactcag
4681 tgatcgactg caacacatgt gtcacccaga cagtcgattt cagcttggat cccaccttca
4741 ccattgagac gacaacgctg ccccaagacg cggtgtcgcg tgcgcagcgg cgaggtagga
4801 ctggcagggg caggagtggc atctacaggt ttgtgactcc aggagaacgg ccctcaggca
4861 tgttcgactc ctcggtcctg tgtgagtgct atgacgcagg ctgcgcttgg tatgagctca
4921 cgcccgctga gacctcggtt aggttgcggg cttacctaaa tacaccaggg ttgcccgtct
4981 gccaggacca cctagagttc tgggagagcg tcttcacagg cctcacccac atagatgccc
5041 acttcttgtc ccagaccaaa caggcaggag acaacctccc ctacctggta gcataccaag
5101 ccacagtgtg cgccagggct caggctccac ctccatcgtg ggaccaaatg tggaagtgtc
5161 tcatacggct aaagcccaca ctgcatgggc caacgcccct gctgtacagg ctaggagccg
5221 ttcaaaatga ggtcactctc acacacccca taaccaaata catcatggca tgcatgtcgg
5281 ctgacctgga ggtcgtcact agcacctggg tgctagtagg cggagtcctt gcggctctgg
5341 ccgcgtactg cctgacgaca ggcagcgtgg tcattgtggg caggatcatc ttgtccggga
5401 ggccagctgt tattcccgac agggaagtcc tctaccagga gttcgatgag atggaagagt

5461 gtgcttcaca cctcccttac atcgagcaag gaatgcagct cgccgagcaa ttcaaacaga
5521 aggcgctcgg attgctgcaa acagccacca agcaagcgga ggctgctgct cccgtggtgg
5581 agtccaagtg gcgagccctt gaggtcttct gggcgaaaca catgtggaac ttcatcagcg
5641 ggatacagta cttggcaggc ctatccactc tgcctggaaa ccccgcgata gcatcattga
5701 tggcttttac agcctctatc accagcccgc tcaccaccca aaataccctc ctgtttaaca
5761 tcttgggggg atgggtggct gcccaactcg ctcccccag cgctgcttcg gctttcgtgg
5821 gcgccggcat tgccggtgcg gccgttggca gcataggtct cgggaaggta cttgtggaca
5881 ttctggcggg ctatggggcg ggggtggctg gcgcactcgt ggcctttaag gtcatgagcg
5941 gcgagatgcc ctccactgag gatctggtta atttactccc tgccatcctt tctcctggcg
6001 ccctggttgt cggggtcgtg tgcgcagcaa tactgcgtcg gcacgtgggc ccgggagagg
6061 gggctgtgca gtggatgaac cggctgatag cgttcgcttc gcggggtaac cacgtctccc
6121 ccacgcacta tgtgcccgag agcgacgccg cggcgcgtgt tactcagatc ctctccagcc
6181 ttaccatcac tcagttgctg aagaggcttc atcagtggat taatgaggac tgctccacgc
6241 cttgttccgg ctcgtggcta aaggatgttt gggactggat atgcacggtg ttgagtgact
6301 tcaagacttg gctccagtcc aagctcctgc cgcggttacc gggactccct ttcctgtcat
6361 gccaacgcgg gtacaaggga gtctggcggg gggatggcat catgcaaacc acctgcccat
6421 gtggagcaca gatcaccgga catgtcaaaa atggctccat gaggattgtt gggccaaaaa
6481 cctgcagcaa cacgtggcat ggaacattcc ccatcaacgc atacaccacg ggcccctgca
6541 cgccctcccc agcgccgaac tattccaggg cgctgtggcg ggtggctgct gaggagtacg
6601 tggaggttac gcgggtgggg gatttccact acgtgacggg catgaccact gacaacgtga
6661 aatgcccatg ccaggttcca gcccctgaat ttttcacgga ggtggatgga gtacggttgc
6721 acaggtatgc tccagtgtgc aaacctctcc tacgagagga ggtcgtattc caggtcgggc
6781 tcaaccagta cctggtcggg tcacagctcc catgtgagcc cgaaccggat gtggcagtgc
6841 tcacttccat gctcaccgac ccctctcata ttacagcaga gacggccaag cgtaggctgg
6901 ccagggggtc tcccccctcc ttggccagct cttcagctag ccagttgtct gcgccttctt
6961 tgaaggcgac atgtactacc catcatgact ccccggacgc tgacctcatc gaggccaacc
7021 tcctgtggcg gcaggagatg ggcgggaaca tcacccgtgt ggagtcagaa aataaggtgg
7081 taatcctgga ctctttcgat ccgattcggg cggtggagga tgagagggaa atatccgtcc
7141 cggcggagat cctgcgaaaa cccaggaagt tcccccagc gttgcccata tgggcacgcc
7201 cggattacaa ccctccactg ctagagtcct ggaaggaccc ggactacgtc cccccggtgg
7261 tacacgggtg ccctttgcca tctaccaagg cccccccaat accacctcca cggaggaaga
7321 ggacggttgt cctgacagag tccaccgtgt cttctgcctt ggcggagctc gctactaaga
7381 cctttggcag ctccgggtcg tcggccgttg acagcggcac ggcgactggc cctcccgatc
7441 aggcctccga cgacggcgac aaaggatccg acgttgagtc gtactcctcc atgcccccc
7501 tcgagggaga gccaggggac cccgacctca gcgacgggtc ttggtctacc gtgagcgggg

7561 aagctggtga ggacgtcgtc tgctgctcaa tgtcctatac atggacaggt gccttgatca
7621 cgccatgcgc tgcggaggag agcaagttgc ccatcaatcc gttgagcaac tctttgctgc
7681 gtcaccacag tatggtctac tccacaacat ctcgcagcgc aagtctgcgg cagaagaagg
7741 tcacctttga cagactgcaa gtcctggacg accactaccg ggacgtgctc aaggagatga
7801 aggcgaaggc gtccacagtt aaggctaggc ttctatctat agaggaggcc tgcaaactga
7861 cgcccccaca ttcggccaaa tccaaatttg gctacggggc gaaggacgtc cggagcctat
7921 ccagcagggc cgtcaaccac atccgctccg tgtgggagga cttgctggaa gacactgaaa
7981 caccaattga taccaccatc atggcaaaaa atgaggtttt ctgcgtccaa ccagagaaag
8041 gaggccgcaa gccagctcgc cttatcgtat tcccagacct gggggtacgt gtatgcgaga
8101 agatggccct ttacgacgtg gtctccaccc ttcctcaggc cgtgatgggc ccctcatacg
8161 gattccagta ctctcctggg cagcgggtcg agttcctggt gaatacctgg aaatcaaaga
8221 aatgccctat gggcttctca tatgacaccc gctgctttga ctcaacggtc actgagaatg
8281 acatccgtac tgaggaatca atttaccaat gttgtgactt ggcccccgaa gccaggcagg
8341 ccataaggtc gctcacagag cggctttatg tcgggggtcc cctgactaat tcgaaggggc
8401 agaactgcgg ttatcgccgg tgccgcgcaa gtggcgtgct gacgactagc tgcggcaaca
8461 ccctcacatg ttacttgaag gccactgcgg cctgtcgagc tgcaaagctc caggactgca
8521 cgatgctcgt gaacggagac gaccttgtcg ttatctgtga gagtgcggga acccaggagg
8581 atgcggcggc cctacgagcc ttcacggagg ctatgactag gtattccgcc ccccccgggg
8641 acccgcccca accagaatac gacttggagc tgataacgtc atgctcctcc aatgtgtcgg
8701 tcgcgcacga tgcatccggc aaaagggtgt actacctcac ccgtgacccc accacccccc
8761 tcgcacgggc tgcgtgggag acagttagac acactccagt caactcctgg ctaggcaata
8821 tcatcatgta tgcgcccacc ctatgggcga ggatgattct gatgactcat ttcttctcta
8881 tccttctagc tcaggagcaa cttgaaaaag ccctggattg tcagatctac ggggcctgtt
8941 actccattga gccacttgac ctacctcaga tcattgaacg actccatggt cttagcgcat
9001 tttcactcca cagttactct ccaggtgaga tcaatagggt ggcttcatgc ctcaggaaac
9061 ttggggtacc gcctttgcga gtctggagac atcgggccag aagtgtccgc gctaagctac
9121 tgtcccaggg ggggagggct gccacttgcg gcaagtacct cttcaactgg gcagtaaaga
9181 ccaagcttaa actcactcca atcccggctg cgtcccagct agacttgtcc ggctggttcg
9241 ttgctggtta caacggggga gacatatatc acagcctgtc tcgtgcccga ccccgttggt
9301 tcatgttgtg cctactccta ctttctgtag ggtaggcat ctacctgctc cccaaccggt
9361 gaacggggag ctaaccactc caggccaata ggccattccc ttttttttt ttc (SEQ ID NO: 17)

General Target Region:

5' Untranslated Region - nts 1 - 328 - Internal Ribosome Entry Site (IRES):

5'UUGGGGGCGACACUCCACCAUAGAUCACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUUGUGCAGCCUCCAGGACCCCCCCUCCCGGGAGAGCCAUAGUGGUCUGCGGAACCGGUGAGUACACCGGAAUUGCCAGGACGACCGGGUCCUUUCUUGGAUCAACCCGCUCAAUGCCUGGAGAUUUGGGCGUGCCCCCGCGAGACUGCUAGCCGAGUAGUGUUGGGUCGCGAAAGGCCUUGUGGUACUGCCUGAUAGGGUGCUUGCGAGUGCCCCGGGAGGUCUCGUAGACCGUGCAU3' (SEQ ID NO: 18)

Initial Specific Target Motifs:

(1) Subdomain IIIc within HCV IRES - nts 213 - 226
5'AUUUGGGCGUGCCC3' (SEQ ID NO: 19)

(2) Subdomain IIId within HCV IRES - nts 241-267
5'GCCGAGUAGUGUUGGGUCGCGAAAGGC3' (SEQ ID NO: 20)

5.8. Ribonuclease P RNA ("RNaseP")

GenBank Accession #s

X15624 Homo sapiens RNaseP H1 RNA:

1 atgggcggag ggaagctcat cagtggggcc acgagctgag tgcgtcctgt cactccactc
61 ccatgtccct tgggaaggtc tgagactagg gccagaggcg gccctaacag ggctctccct
121 gagcttcagg gaggtgagtt cccagagaac ggggctccgc gcgaggtcag actgggcagg
181 agatgccgtg gaccccgccc ttcggggagg ggcccggcgg atgcctcctt tgccggagct
241 tggaacagac tcacggccag cgaagtgagt tcaatggctg aggtgaggta ccccgcaggg
301 gacctcataa cccaattcag accactctcc tccgcccatt (SEQ ID NO: 21)

U64885 Staphylococcus aureus RNaseP (rrnB) RNA:

1 gaggaaagtc cgggctcaca cagtctgaga tgattgtagt gttcgtgctt gatgaaacaa
61 taaatcaagg cattaatttg acggcaatga aatatcctaa gtctttcgat atggatagag
121 taatttgaaa gtgccacagt gacgtagctt ttatagaaat ataaaaggtg gaacgcggta
181 aacccctcga gtgagcaatc caaatttggt aggagcactt gtttaacgga attcaacgta
241 taaacgagac acacttcgcg aaatgaagtg gtgtagacag atggttatca cctgagtacc
301 agtgtgacta gtgcacgtga tgagtacgat ggaacagaac gcggcttat (SEQ ID NO: 22)

M17569 Escherichia coli RNA component (M1 RNA) of ribonuclease P (rnpB) gene:

1 gaagctgacc agacagtcgc cgcttcgtcg tcgtcctctt cgggggagac gggcggaggg

61 gaggaaagtc cgggctccat agggcagggt gccaggtaac gcctgggggg gaaacccacg
121 accagtgcaa cagagagcaa accgccgatg gcccgcgcaa gcgggatcag gtaagggtga
181 aagggtgcgg taagagcgca ccgcgcggct ggtaacagtc cgtggcacgg taaactccac
241 ccggagcaag gccaaatagg ggttcataag gtacggcccg tactgaaccc gggtaggctg
301 cttgagccag tgagcgattg ctggcctaga tgaatgactg tccacgacag aacccggctt
361 atcggtcagt ttcacct (SEQ ID NO: 23)

Z70692 Mycobacterium tuberculosis RNaseP (rnpB) RNA:
1 ccaccggtta cgatcttgcc gaccatggcc ccacaatagg gccgggggaga cccggcgtca
61 gtggtgggcg gcacggtcag taacgtctgc gcaacacggg gttgactgac gggcaatatc
121 ggctccatag cgtcggccgc ggatacagta aaggagcatt ctgtgacgga aaagacgccc
181 gacgacgtct tcaaacttgc caaggacgag aaggtcgaat atgtcgacgt ccggttctgt
241 gacctgcctg gcatcatgca gcacttcacg attccggctt cggcctttga caagagcgtg
301 tttgacgacg gcttggcctt tgacggctcg tcgattcgcg ggttccagtc gatccacgaa
361 tccgacatgt tgcttcttcc cgatcccgag acggcgcgca tcgacccgtt ccgcgcggcc
421 aagacgctga atatcaactt ctttgtgcac gacccgttca ccctggagcc gtactcccgc
481 gacccgcgca acatcgcccg caaggccgag aactacctga tcagcactgg catcgccgac
541 accgcatact tcggcgccga ggccgagttc tacattttcg attcggtgag cttcgactcg
601 cgcgccaacg gctccttcta cgaggtggac gccatctcgg ggtggtggaa caccggcgcg
661 gcgaccgagg ccgacggcag tcccaaccgg ggctacaagg tccgccacaa gggcgggtat
721 ttcccagtgg cccccaacga ccaatacgtc gacctgcgcg acaagatgct gaccaacctg
781 atcaactccg gcttcatcct ggagaagggc caccacgagg tgggcagcgg cggacaggcc
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5.9. X-linked Inhibitor of Apoptosis Protein ("XIAP")

GenBank Accession # U45880:

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Initial Specific Target Motif:

RNP core binding site within XIAP IRES

5'GGAUUUCCUAAUAUAAUGUUCUCUUUUU3' (SEQ ID NO: 27)

5.10. Survivin

GenBank Accession # NM_001168:

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

The invention can be illustrated by the following embodiments enumerated in the numbered paragraphs that follow:

1. A method for identifying a test compound that binds to a target RNA molecule, comprising the steps of (a) contacting a detectably labeled target RNA molecule with a library of solid support-attached test compounds under conditions that permit direct binding of the labeled target RNA to a member of the library of solid support-attached test compounds so that a detectably labeled target RNA:support-attached test compound complex is formed; (b) separating the detectably labeled target RNA:support-attached test compound complex formed in step (a) from uncomplexed target RNA molecules and test compounds, and (c) determining a structure of the test compound of the RNA:support-attached test compound complex.

2. The method of paragraph 1 in which the target RNA molecule contains an HIV TAR element, internal ribosome entry site, "slippery site", instability element, or adenylate uridylate-rich element.

3. The method of paragraph 1 in which the RNA molecule is an element derived from the mRNA for is tumor necrosis factor alpha ("TNF-α"), granulocyte-macrophage colony stimulating factor ("GM-CSF"), interleukin 2 ("IL-2"), interleukin 6 ("IL-6"), vascular endothelial growth factor ("VEGF"), human immunodeficiency virus I ("HIV-1"), hepatitis C virus ("HCV" - genotypes 1a & 1b), ribonuclease P RNA ("RNaseP"), X-linked inhibitor of apoptosis protein ("XIAP"), or survivin.

4. The method of paragraph 1 in which the detectably labeled RNA is labeled with a fluorescent dye, phosphorescent dye, ultraviolet dye, infrared dye, visible dye, radiolabel, enzyme, spectroscopic colorimetric label, affinity tag, or nanoparticle.

5. The method of paragraph 1 in which the test compound is selected from a combinatorial library comprising peptoids; random bio-oligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; and small organic molecule libraries including, but not limited to, benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, or diazepindiones.

6. The method of paragraph 1 in which screening a library of test compounds preferably comprises contacting the test compound with the target nucleic acid in the presence of an aqueous solution, the aqueous solution comprising a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions

7. The method of paragraph 6 in which the aqueous solution optionally further comprises non-specific nucleic acids comprising DNA, yeast tRNA, salmon sperm DNA, homoribopolymers, and nonspecific RNA.

8. The method of paragraph 6 in which the aqueous solution further comprises a buffer, a combination of salts, and optionally, a detergent or a surfactant. In another embodiment, the aqueous solution further comprises a combination of salts, from about 0 mM to about 100 mM KCl, from about 0 mM to about 1 M NaCl, and from about 0 mM to about 200 mM $MgCl_2$. In a preferred embodiment, the combination of salts is about 100 mM KCl, 500 mM NaCl, and 10 mM $MgCl_2$. In another embodiment, the solution optionally comprises from about 0.01% to about 0.5% (w/v) of a detergent or a surfactant.

9. Any method that detects an altered physical property of a target nucleic acid complexed to a test compound attached to a solid support from the unbound target nucleic acid may be used for separation of the complexed and non-complexed target nucleic acids in the method of paragraph 1. Methods such as flow cytometry, affinity chromatography, manual batch mode separation, suspension of beads in electric fields, and microwave are used for the separation of the complexed and non-complexed target nucleic acids.

10. The structure of the substantially one type of test compound of the RNA:test compound complex of paragraph 1 is determined, in part, by the type of library of test compounds. In a preferred embodiment wherein the combinatorial libraries are small organic molecule libraries, mass spectroscopy, NMR, or vibration spectroscopy are used to determine the structure of the test compounds. In an embodiment wherein the combinatorial libraries are peptide or peptide-based libraries, Edman degradation is used to determine the structure of the test compounds.

WHAT IS CLAIMED IS:

1. A method for identifying a test compound that binds to a target RNA molecule, comprising the steps of:

(a) contacting a detectably labeled target RNA molecule with a library of solid support-attached test compounds under conditions that permit direct binding of the labeled target RNA to a member of the library of solid support-attached test compounds so that a detectably labeled target RNA:support-attached test compound complex is formed;

(b) separating the detectably labeled target RNA:support-attached test compound complex formed in step (a) from uncomplexed target RNA molecules and test compounds by flow cytometry; and

(c) determining a structure of the substantially one type of test compound of the RNA:support-attached test compound complex by mass spectroscopy.

SEQUENCE LISTING

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<120> METHODS FOR IDENTIFYING SMALL MOLECULES THAT BIND SPECIFIC RNA STRUCTURAL MOTIFS

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<140> To be assigned

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/11758

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12M 1/38, 1/40; C12Q 1/68
US CL :435/6, 91.2, 172.3, 286.1, 286.5, 282.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 172.3, 286.1, 286.5, 282.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST: USPAT, DERWENT/EP ABSTRACT.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,060,240 A(KAMB et al.) 09 May 2000, see entire document.	1
Y	5,716,825A (HANCOCK et al.) 10 February 1998, see entire document, especially columns 7-8.	1
A	US 5,667,975 A (DYKSTRA et al.) 16 September 1997, see entire document.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 JUNE 2002	18 SEP 2002

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